

AN ABSTRACT OF THE THESIS OF

Sylvia E. Meadows for the degree of Doctor of Philosophy in  
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Title: Phenolic Acids and Ethylene Biosynthesis in Pears During  
Ripening and Storage

Abstract approved: \_\_\_\_\_

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Fruit flesh tissue of mature 'Beurre d'Anjou' and 'Beurre Bosc' (Pyrus communis L.) contained as the major free phenolic acids in order of descending quantity: chlorogenic acid, catechin, arbutin, epicatechin and p-coumaric acid derivatives. At harvest 'd'Anjou' pears contained 50 ug/g fresh weight chlorogenic acid, 13 ug/g catechin, 10 ug/g arbutin and no detectable epicatechin or p-coumaroyl quinate. At harvest 'Bosc' pears contained 50 ug/g chlorogenic acid, 14 ug/g catechin, 19 ug/g arbutin, and no detectable epicatechin or p-coumaroyl quinate. Epicatechin, p-coumaroyl quinate and a p-coumaric acid derivative appeared with the onset of ethylene production, however, exogenously applied phenolics to fruit tissue plugs did not stimulate ethylene production. Chlorogenic acid, catechin, epicatechin and p-coumaric acid derivatives increased in 'd'Anjou' with duration in

-1.1°C storage to 70, 30, 18 and 1.5 ug/g fresh weight, respectively.

Winter pears, including 'd'Anjou', require storage at -1° C before they can ripen satisfactorily at 20°. Mature 'd'Anjou' pear fruits require about 50 days in order to initiate ethylene production and fruit softening. Exogenous ethylene prior to completion of the cold requirement can cause respiration rate to double, extractable juice to decrease, and fruit firmness to decrease from 66 Newtons to 10 Newtons over a 12 day period. Taste panelists rated ethylene treated fruit as ripe, sweet, and juicy, while untreated fruit was rated comparably to unripened fruit. 'd'Anjou' pear flesh discs were unable to convert added 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene after 21 days cold storage, but were able to after 41 and 50 days. Protein synthesis is apparently required for the synthesis of ACC and conversion of ACC to ethylene. Pear discs given 0.5 mM ACC and 0.05 mM cycloheximide produced twice as much ethylene as untreated discs, whereas discs with ACC but no cycloheximide produced 4 times the amount of ethylene as discs with ACC and cycloheximide. 'd'Anjou' fruit stored at -1° for 125 days in 3% O<sub>2</sub> or 153 days at 1% O<sub>2</sub> had internal ethylene levels equal to air stored fruit, but had ACC levels 20 times higher. Discs from air stored fruit could produce 400 times the amount of ethylene as 1% O<sub>2</sub> stored fruit, when given 0.05 mM cycloheximide and 0.5 mM ACC.

Phenolic Acids and Ethylene Biosynthesis  
in Pears during Ripening and Storage

by

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## PREFACE

This thesis is presented as a series of three papers written in the format required by the Journal of the American Society for Horticultural Science.

# PHENOLIC ACIDS AND ETHYLENE BIOSYNTHESIS IN PEARS DURING RIPENING AND STORAGE

## CHAPTER I.

### INTRODUCTION

Biochemical changes, particularly those involving ethylene , that occur in pears during ripening and long-term cold storage are of interest in that they may offer a mechanism to extend the useful storage life of pears. Study of the postharvest life of pears could potentially increase the quantity and quality of pears for human consumption as well as assist in more orderly marketing.

Phenolic acids in plants have been known for years because of their participation in browning reactions of plants. In more recent years they have also been shown to have roles in other aspects of plant physiology such as in enzyme regulation, fruit astringency, disease resistance, cell wall lignin synthesis and regulation of growth and development.

Ethylene, classed as a plant hormone, is a hydrocarbon gas produced naturally by pears and many other climacteric fruits. The presence of ethylene in sufficient quantities causes ripening and decreases the storage life of pears.

Both phenolic acids and ethylene can affect the postharvest quality of pears. There have been implications that phenolic acids and ethylene biosynthesis, in addition to affecting pear quality separately, may have regulatory effects on each other.

This thesis examines changes that occur in phenolic acids and ethylene biosynthesis under different ripening and storage situations and relates them to other postharvest characteristics of pears. The possibility of phenolic acid and ethylene interactions are also discussed in relation to storage life.

The first manuscript (Chapter III) describes changes that occur in the levels of phenolic acids during long-term cold storage of pears. These changes are discussed in relation to the initiation of ethylene biosynthesis and the possible influence of phenolic acids on ethylene production.

The second manuscript (Chapter IV) describes changes in quality and ethylene biosynthesis that occur during the cold requirement for ripening in 'd'Anjou' pears, and the effects of exogenous ethylene during the cold requirement. The first objective of this manuscript was to examine biochemical changes that occur in pears as the result of ethylene exposure and to see to what extent quality, as perceived by a taste panel, was altered. The second objective was to characterize the development of the ability to produce ethylene during the cold period.

The third manuscript (Chapter V) deals with the development of the ethylene biosynthetic pathway over the storage life of pears, both in air and controlled atmosphere storages. The

effects of controlled atmosphere storages on 1-aminocyclopropane-1-carboxylic acid (ACC) concentrations and ethylene concentrations are discussed in relation to the beneficial effects of controlled atmosphere storages.

## CHAPTER II.

### LITERATURE REVIEW

#### ETHYLENE IN PLANTS

Ethylene is a hydrocarbon gas that is produced naturally by plant tissues which has the ability to elicit physiological and biochemical responses in plants. Many fruits are induced to ripen and senesce by ethylene. Ethylene's ability to induce ripening and senescent changes in fruit has made it of interest to horticulturists as a possible point of postharvest control to prolong storage of fruit. Characterization and control of the ethylene biosynthetic pathway offers a mechanism to reduce losses in storage of horticultural commodities.

#### BIOSYNTHESIS OF ETHYLENE

##### Methionine as a Precursor

During the past ten years the pathway for ethylene biosynthesis has been clarified and defined substantially. The biosynthesis of ethylene and its consequences have been reviewed (3,142,257). Through a series of experiments by several groups of researchers it has been established that methionine is the major precursor of ethylene in higher plants. Carbons 3 and 4 of methionine have been shown to be incorporated into ethylene in vegetative and fruit tissue (28,41,91,144,205). Methionine was



also found to be the precursor of wound ethylene (2,90). Burg and Clagett (41) found during the breakdown of methionine that carbon 1 of methionine formed  $\text{CO}_2$ . Formic acid was made from carbon 2 and the methyl thio group was recycled intact.

In the past numerous other substances have been considered as possible precursors of ethylene. Homocysteine and homoserine were once thought to be direct ethylene precursors. However, it has now been proposed that although these amino acids serve to increase certain methionine pool levels, methionine is a more direct precursor to ethylene (28,91).

Linolenic acid,  $\alpha$ -keto- $\gamma$ -methylthiobutyric acid, and methional were all variously shown to be involved as precursors to ethylene production (28,151). S-methylmethionine was also shown to be a better precursor to ethylene than methionine (90,91,205).

In a model system for ethylene production peptides containing methionine were shown to act as precursors for ethylene with a C-terminal methionine being more readily utilized than an N-terminal or internal methionine (59). In labeling studies with  $^{14}\text{C}$ -methionine, Konze et.al. (130), found in pea stems and flowers that unlabeled selenomethionine and selenoethionine reduced the amount of specific radioactivity that appeared in ethylene from  $^{14}\text{C}$  methionine. Apparently methionine derivatives can act as competing substrates in the ethylene pathway.

Direct application of unlabelled methionine to plant tissues has provided little evidence for its conversion to ethylene. In a variety of vegetative tissues, 1 mM methionine was found to have

little effect on ethylene production (42). In Atropa belladonna callus culture added methionine decreased ethylene production (224). However, with post-climacteric apple fruit, application of methionine has given a 100% increase in ethylene, yet does not increase ethylene by young, pre-climacteric or climacteric fruit (144).

Model systems have been devised to study ethylene production.  $\text{Cu}^{++}$  and ascorbate,  $\text{Cu}^{++}$ ,  $\text{Fe}^{++}$ , and  $\text{H}_2\text{O}_2$  catalyze non-enzymatic ethylene production. Methional and 2-keto-4-methylthiobutyric acid can serve as substrates for peroxidase to generate ethylene (3). Further support for methionine as the major precursor was that ethylene was the only hydrocarbon evolved from methionine whereas other model systems evolved several other hydrocarbon gases including ethane (144). Isolated chloroplasts and etioplasts produced ethylene from methionine when supplied with pyridoxal-phosphate, ferredoxin, and NADPH. In early work with a model system of cauliflower florets, two enzyme systems were implicated in the conversion of methional to ethylene. The first enzyme was an oxidase which generated hydrogen peroxide, and a second enzyme that used cofactors and peroxide (153). In the horseradish peroxidase system converting methional to ethylene, it was found that monohydroxy phenols activated the conversion while it was inhibited by o-dihydroxy phenols. Chlorogenic acid and catechol were inhibitory while tyrosine and resorcinol were most promotive (256). Dihydroxy phenols have an inhibitory effect on the

methional to ethylene system in aqueous extracts of cauliflower florets (153). Catechol, caffeic and ferulic acids have the greatest inhibitory effects.

Despite the fact that methionine has been widely accepted as the precursor to ethylene production in higher plants, there may be some exceptions. Bacteria and fungi can utilize other precursors and pathways. 4-methylthio-2-oxobutanoate can be an intermediate in addition to methionine for ethylene (33) in bacteria and fungi, and depending on growing conditions Penicillium digitatum can produce ethylene by several pathways using  $\alpha$ -ketoglutarate and glutamate as precursors (161).

In higher plants there has been some evidence that ethylene production may proceed by a different pathway depending on the age of the fruit. With the ethylene inhibitor aminoethoxyvinylglycine (AVG), sensitivity to AVG in tomatoes declines as the fruit ages indicating that there may be an alternate pathway as AVG blocks ethylene synthesis from methionine (27). Rhizobitoxine inhibited the conversion of methionine to ethylene in unripe and ripe tomatoes. In unripe tomatoes the conversion was inhibited by 60-70%. When the tomatoes turned pink and reached the soft-ripe stage, rhizobitoxine inhibited ethylene production by only 12%. When tomato slices were fed  $^{14}\text{C}$ -methionine, rhizobitoxine inhibited  $^{14}\text{C}$ -ethylene production. The authors concluded that tomatoes used precursors other than methionine for ethylene production at some points in development.

Avocadoes may have an alternate pathway. AVG has little effect on avocadoes (25) and the ability to produce ethylene in the presence of AVG has yet to be explained. Avocadoes can't utilize methionine in a pre-climacteric state, although they can during the climacteric rise and peak (29). Based on changes in the ratios of  $C^{13}/C^{12}$  during different stages of development, other work with avocadoes has suggested that amino acids may be the precursors for ethylene during the early climacteric rise and as the climacteric progresses fatty acids become the precursors (142).

#### S-adenosylmethionine as an Intermediate

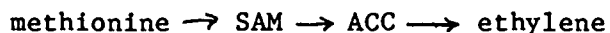
After methionine was established as one of the precursors to ethylene, researchers began looking for other intermediates in the conversion of methionine to ethylene and the enzymes involved. In a model system, Mapson, March and Wardale (152) found in cauliflower florets that a transaminase was necessary for the conversion of methionine to ethylene. The transaminase was shown to form 4-methylmercapto-2-oxobutyric acid. When this oxo acid was labeled with  $^{14}C$  the radioactivity appeared in ethylene. Baur and Yang (28) showed that the alpha-keto analog of methionine was not an intermediate as Mapson claimed but was produced by extracellular reactions. Lieberman and Kunishi (144) found in cauliflower florets that 4-S-methyl-2-keto-butyric acid was not an intermediate in ethylene biosynthesis but its formation was due to disruption of the tissue. It was postulated that in senescing

morning-glory rib segments that S-methyl methionine was involved as an intermediate between methionine and ethylene. Later it was proven that S-methyl methionine was not involved (205).

S-adenosyl methionine (SAM) was later shown to be an intermediate in ethylene biosynthesis (9,10). A comprehensive review of S-adenosyl methionine synthesis and metabolic and regulatory function in animals has been compiled (147). In animals a similar pathway exists with 1-amino-cyclopentane carboxylic acid acting as an inhibitor of methionine conversion with SAM synthesis being self-regulating (147).

#### ACC as an Intermediate

It was then found that when apple tissue was fed  $^{14}\text{C}$ -methionine and held in nitrogen atmosphere that labelled 1-aminocyclopropane-1-carboxylic acid (ACC) accumulated. When ACC was fed to apple tissue ethylene was produced (12,13) or when the tissue was removed from the nitrogen atmosphere the accumulated ACC was converted to ethylene. This also proved that the conversion of ACC to ethylene required  $\text{O}_2$  (11,12). When a variety of vegetative and reproductive tissues were treated with ACC there were increases in ethylene (42). An assay was developed for measuring ACC levels in plants (146). Through the aforementioned work, the pathway below was established:



### Recycling of Sulfur

While part of SAM is converted to ACC to produce ethylene, the remainder of the molecule is recycled back to methionine. This pathway is believed to exist as a way of conserving sulfur in the plant (257). Baur, Yang, and Pratt (29) suggested that must be the case because endogenous levels of methionine were so low that it must be recycled in order to account for the high rates of ethylene synthesis. It was originally thought that the  $\text{CH}_3\text{-S}$  group of methionine was first incorporated into S-methylcysteine. S-methylcysteine was then converted to cysteine. Cysteine donated its sulfur to methionine via cystathione and homocysteine. Later it was realized that SAM was converted to methylthioadenosine (MTA) and then by way of MTA nucleosidase to methylthioribose (MTR) before further changes back to methionine (10). Methylthioribose kinase, which converts MTR to MTR-1-phosphate has been identified in tomato, pear, avocado, apple and strawberry. MTR-1-phosphate is then possibly converted to methionine (133). Whether this ATP-requiring step is critical to methionine recycling is currently being investigated.

### Enzymes and Control of Biosynthesis

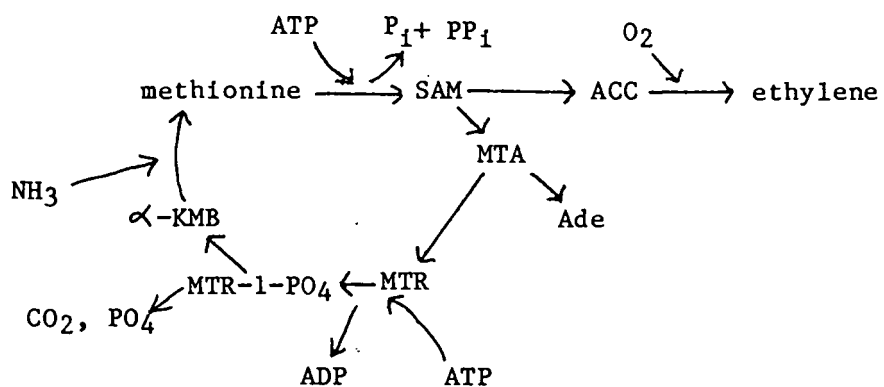
ACC synthase, which converts SAM to ACC, has been isolated (36,258). It was found to have a pH optimum of 8.5 and is probably a pyridoxal enzyme (258). In addition, Adams and Yang (12) found that the AVG sensitive step in the ethylene pathway is the conversion of SAM to ACC, by ACC synthase (12). ACC synthase

is neither promoted nor inhibited by ACC or ethylene in wounded cantaloupe (107). It was thought that a peroxidase caused the conversion of ACC to ethylene. However, it's been shown that peroxidase does not directly cause a decarboxylation of ACC or ring cleavage, but may provide radicals,  $H_2O_2$  or other unknown factors (196).

The development of the ethylene system during plant development has been studied. Pre-climacteric and climacteric apple fruit can convert methionine to SAM. However, pre-climacteric apples are unable to produce ethylene even if given ACC. Vegetative tissues can produce large amounts of ethylene when given ACC (53). In most plant systems ethylene production is limited by the availability of ACC (18).

Free radicals may be involved in ethylene biosynthesis. In chloroplasts and etioplasts in the light or dark, with NADPH as an electron donor, ethylene can be produced. Inhibition by catalase, superoxide dismutase and ascorbate implicate a  $OH^-$  radical (129).

The currently accepted pathway of ethylene biosynthesis in plants is as follows:





## PROMOTERS AND INHIBITORS OF ETHYLENE BIOSYNTHESIS

Organic Inhibitors

Organic, inorganic and environmental parameters can influence ethylene biosynthesis in plant tissues. Aminoethoxyvinylglycine (AVG), aminoxyacetic acid (AOA) and rhizobitoxine analogs have been shown to inhibit ethylene production (26,27,90,159,258). AVG has been shown to also inhibit protein synthesis (159). AVG and AOA inhibit the action of ACC synthase (258). Inhibition by rhizobitoxine analogs is dependent on the age of the tissue, inhibiting ethylene strongly in green tomatoes but only slightly in pink and red tomatoes (26,27). Preharvest applications of AVG inhibits ethylene in pears (179,197,239) and apples (38) during storage.

Cycloheximide inhibits chemical-stress-induced ethylene and auxin-induced ethylene (2,255). Actinomycin D also inhibits auxin-induced ethylene (255).

2,4-Dinitrophenol, other uncouplers of oxidative phosphorylation, reducing agents, and inhibitors of electron transfer inhibit ethylene production in plants (18,20). The step(s) of ACC to ethylene are not effected by pyridoxal phosphate inhibitors (18).

Free radical scavengers , such as propyl gallate and benzoic acid inhibit ethylene production in several plant tissues (18,20,26). It is believed that free radicals may be involved between of ACC and ethylene. 3-Hydroxytyramine (dopamine) has

been shown to stimulate ethylene production in sugar beet leaves by stimulating superoxide free radicals via phenoloxidase (68).

Phenothiazines inhibit ACC synthase in tomatoes, thereby lowering ethylene synthesis (158).

#### Organic Promoters

Benzohydroxamic acid and 3-chlorobenzohydroxamic acid promote ethylene production in the presence or absence of ACC in cocklebur cotyledons (204). Auxin-induced ethylene is well known in plants, especially vegetative tissues. In mung bean hypocotyls auxin regulates ethylene synthesis at the conversion of SAM to ACC (259,260). Auxin inhibits ripening has been shown in 'Bartlett' pears (76).

#### Inorganic Promoters and Inhibitors

Inorganic ions also affect ethylene biosynthesis. Cytokinins and  $\text{Ca}^{++}$  increased ethylene in mung beans (257), however  $\text{Ca}^{++}$  is generally thought to reduce ethylene synthesis (185).

In studies with subcellular fractions of tomato (163,164,165) it was felt that ethylene production required sulfhydryl groups, transamination and possibly a metalloprotein or cation. Cobalt reduced ethylene production in subcellular fractions. Cobalt blocked the conversion of ACC to ethylene in other plants (136). Silver ions inhibited ethylene production in fruit (116) and in vegetative tissues (15,32). Copper chelators blocked ethylene in

apple tissue slices (144). Phosphate level may regulate ethylene production in Penicillium digitatum (161).

#### Naturally Occurring Promoters and Inhibitors

Some naturally occurring plant products have been shown to influence ethylene production along with environmental factors. In seed tissues the D-amino acids of phenylalanine, valine, leucine, threonine, methionine and ethionine stimulated ethylene production while the L-isomers did not (203).

Inhibitors from tomato extract decreased ethylene synthesis using  $\alpha$ -keto- $\gamma$ -methylthiobutyrate as a substrate (132). the inhibitors were thought to be phenolic in nature as catechol, caffeic acid, chlorogenic acid and quercetin inhibited the reaction. p-Coumaric acid stimulated the system.

The short chain fatty acids, caprylic acid and capric acid, inhibited the conversion of ACC to ethylene in a variety of fruits and vegetables. Fatty acids are presumed to interfere with membrane function (112).

Polyamines, formed via an alternate pathway from SAM, inhibited ethylene production in protoplasts and young tissues (19,218).

#### Environmental Promoters and Inhibitors

Light has been shown to influence ethylene in some plants. Mature green tomatoes show a 3-day advance in pigmentation, climacteric rise and ethylene production when exposed to red light

(117). A blue/far red light spectrum increased ethylene in peach apices (70). Conversion of ACC to ethylene was reversibly inhibited by light in tobacco leaves (135) and in water-stressed wheat leaves (253).

Wounding of plant tissue causes a rise in ethylene production, ACC and ACC synthase. ACC synthase regulates the wound ethylene response (37). ACC synthase and the system converting ACC to ethylene are both stimulated by wounding in cantaloupe, and the ACC to ethylene conversion is stimulated by ethylene (107).

Oxygen is essential for conversion of methionine to ethylene (29) or more specifically the steps from ACC to ethylene (257). Plugs of apple tissue held in nitrogen stop producing ethylene but when returned to air produce an ethylene surge due to the build-up of ACC and its subsequent conversion. Base rate ethylene production returns in approximately 25 minutes (11).

Any environmental or chemical effect on the cell wall or membrane appears to affect ethylene production. The cell membrane-cell wall may be the site of ethylene synthesis based on temperature studies in apple and tomato (160). The enzyme that converts ACC to ethylene may be membrane associated. Cold, osmotic shock and surface active agents inhibit this reaction, along with temperatures above 29°C or below 12°C (18). Ethylene was originally thought to be produced by mitochondria (215), but this

has been largely refuted (166). However, ATP produced by mitochondria would be important in the overall pathway, certainly in SAM formation and at the MTR-1-PO<sub>4</sub> step.

Ethylene treated rib segments from morning glory flowers show an increase in membrane permeability and there is some evidence that the tonoplast may be affected (120). The authors felt that this was support for the hypothesis that breakdown of cellular compartmentalization allows mixing of ethylene producing components and this is the basis for ethylene-induced ethylene synthesis (120). Perhaps there is a release or activation of part of the ethylene producing system on the cell wall or membrane. It has been shown that a fungal cell wall degrading mixture promotes ethylene synthesis and ACC formation in tobacco leaf discs (17).

## POSTHARVEST PHYSIOLOGY AND STORAGE LIFE

Pears are stored at  $-1.1^{\circ}\text{C}$  to extend the marketing period. Numerous changes occur in fruit during storage and some of the changes affect the postharvest life of the fruit. Ethylene production, respiration, fruit firmness, and other biochemical components, such as sugar and organic acids levels, affect fruit quality.

'Bosc' pears are a sweet, juicy pear that have a medium storage life, while 'd'Anjou' pears are moderately sweet, slightly tart and are a longer storing winter pear. 'Bosc' internal ethylene and respiration rates were found to be higher than in 'd'Anjou' during storage and ripening (51).

'Bosc' and 'd'Anjou' pears ethanol-insoluble matter, titratable acidity, soluble solids, proteins and free amino acids fluctuated from season to season and couldn't be associated with postharvest life (51).

Softening, increase in soluble pectin, and decrease in extractable juice of apples are all reduced by storage at low temperature (121).

## MODIFIED ATMOSPHERE STORAGE

Controlled atmosphere (CA) and low oxygen storage have been shown to extend the postharvest storage life of pears. The conditions of optimum CA and low oxygen storage in pears have been

studied along with possible effects on quality (54). Commercial CA conditions for 'd'Anjou' pears are 2-2.5% O<sub>2</sub> with 0.8-1.0% CO<sub>2</sub> (89). A prestorage treatment with 12% CO<sub>2</sub> for 14 days improved quality of 'd'Anjou' when followed by regular or CA storage of fruit (58). Levels ranging from 0.5-2% O<sub>2</sub> for low oxygen storage (a special type of CA storage) have been shown to maintain pear quality (54,167). Normally texture, juiciness, and acidity are preserved and aroma is decreased in apples and pears by CA storage (49,141,209).

#### Ethylene Production and Effects

Carbon dioxide (above 3%) and oxygen (below 3%) levels can influence the amount of ethylene produced by plants as well as inhibit the action of ethylene (15,32). 'McIntosh' apples are affected by 10 or 500 ppm ethylene in CA, but not 1 ppm. For 'Delicious', 'Golden Delicious', 'Idared' and 'Cortland' varieties ethylene levels in CA had no effect on soluble solids, titratable acidity, firmness, or quality rating, but high ethylene gave more storage scald (145). A high initial CO<sub>2</sub> treatment in 'd'Anjou' pears delayed the respiratory climacteric and ethylene production, and the beneficial effects of the CO<sub>2</sub> treatment were evident up to 8 months later (238).

Low oxygen (< 1.5%) storage has been found to maintain quality of 'd'Anjou' pears for eight months. Upon subjecting apples to a change in atmosphere, internal atmospheres equilibrated with respect to external O<sub>2</sub> atmospheres in 6-16 hours

(125). Since  $O_2$  is required for the conversion of ACC to ethylene (257), one would expect some equilibration of this system in a similar length of time. However,  $O_2$  less than 0.5% may risk anaerobic metabolic products such as acetaldehyde or ethanol.

Ethylene production of 'Golden Delicious' apples was delayed 35 days in 2%  $O_2$  and 44 days at 5%  $CO_2$  / 3%  $O_2$  (122). Oxygen concentration was inversely related to apple ethylene production (125). When fruit is in a low oxygen atmosphere and the ventilation is such that the atmosphere is product-generated, there is a build-up of ethylene regardless of oxygen concentration. It seems that low oxygen conditions fail to prevent the accumulation of threshold concentrations of ethylene (125).

Propylene <sup>丙烯  $CH_3-CH=CH_2$</sup>  at less than 6.5 %  $O_2$  didn't induce ethylene (207). However an anaerobic atmosphere was necessary to completely inhibit ethylene production once it was initiated (207).

Ethylene (800 ppm) treatment of apple fruit didn't reverse the effects of low oxygen (2%) on ripening processes (124). The effects of low oxygen storage on fruits involves several metabolic aspects rather than simply to control the level of ethylene production (125). Low oxygen storage does however, affect ethylene production. In apple discs held in low oxygen, or cut from fruit held in low oxygen, ethylene induces only a temporary rise in respiration. However, when fruit is placed in oxygen tension equal to that of air, ethylene is quickly stimulated to



the level of fruit held in air. It was surmised that the ethylene pathway can partially proceed in low oxygen but needs high oxygen to complete the synthesis of ethylene (93). Exogenous ethylene in 3% O<sub>2</sub> will have only a temporary effect on apples, however auto-stimulation of ethylene followed by action doesn't seem possible in low oxygen (109). Apparently both ethylene production and action need a relatively high oxygen concentration for operation in apples (109). It should be pointed out that the inhibitory effects of low oxygen on ripening can be overcome by ethylene in late storage but not in early storage (124). In apples as little as 50 ppb ethylene in CA storage has been shown to enhance softening (66).

Apple mitochondria, from fruit stored in 6 to 12 % CO<sub>2</sub>, showed a reduced capacity to oxidize succinate or NADH (210). Therefore one effect of CA may be to reduce adenylate energy charge.

#### Effects of Modified Atmospheres on Softening and Phenolic Acids

Softening reactions are possibly affected by low oxygen storage. 1-2% O<sub>2</sub> slowed softening in apples most during the first 50 days of storage. Oxygen below 21% progressively delayed softening in apples (125). 'Conference' pears, whether stored in air or 2% O<sub>2</sub> had similar changes in softening and pectin (123). CA stored pears had slower general biochemical changes and loss of acids and free amino acids, along with slower fruit softening (167).

Total polyphenols were high in pear flesh before low oxygen storage, and decreased with low oxygen storage (221). Anaerobic storage of pears reduced polyphenol oxidase activity but had no effect on phenylalanine ammonia-lyase (221).

One problem encountered in long term storage of 'd'Anjou' pears is the increased susceptibility of 'd'Anjou' to bruising and scuffing after bulk storage, which limits the packing season (58). CA storage (58) and low oxygen storage (221) reduced severe scuffing compared to regular air storage in 'd'Anjou' (58). Regular CA (2.0-2.5% O<sub>2</sub>, and 0.8-1% CO<sub>2</sub>) maintained 'd'Anjou' pear quality but did not reduce scald. One possible explanation for the reduction would be altered phenolic acid synthesis or polyphenoloxidase activity. Peach and apricot tannins decreased during storage and there was little difference between CA and regular storage (242).

Low oxygen storage delays chlorophyll degradation (167) and inhibits synthesis of lycopene in tomatoes. Lycopene is a plant pigment that is derived in part from the phenolic acids (201). CA stored tomatoes had less synthesis of lycopene, while sugars, organic acids and amino acids changed similarly to ripening fruit (85).

#### BIOCHEMICAL AND PHYSIOLOGICAL CHANGES DURING RIPENING

Ripening in pome fruits entails many biochemical and physiological changes, including changes in respiration and ethylene production. Upon removal from cold storage a small transitory

increase in  $\text{CO}_2$  appears to be common in apples and pears (149). Continued elevated temperatures will induce the respiratory climacteric (124). Protein synthesis must also take place for ripening to occur in pome fruits (77).

Low oxygen stored fruit seem to ripen like fruit stored in air, but exhibit a time lag in the initiation of ripening events. Respiration and ethylene production are similar in whole fruit or discs apples in air, low oxygen, or on transfer from low oxygen to air (109).

#### Ethylene and Fruit Ripening

The synthesis of ethylene during ripening and its action on the other ripening events has been studied extensively in fruit. In apples it appears that something other than a change in the rate of ethylene evolution determines when apples ripen (191). It is felt that ethylene initiates ripening but other unknown factors control the action of ethylene (191). More recently, the rise of internal ethylene concentrations appears promising for predicting ripening ability (66,200). Ethylene synthesis requires protein synthesis at some stages of fruit maturation. Early in the respiratory climacteric, ethylene synthesis can be blocked by cycloheximide, but cycloheximide becomes less effective as the climacteric progressed (77). Conversely, ethylene application cannot overcome the inhibition of ripening by cycloheximide (77). Fruit attached to the tree are less sensitive to ethylene than are

harvested fruit, indicating that an inhibitor may be coming from other parts of the tree (207). This is especially well known in the case of avocado which fails to ripen while still attached (252). Others have felt that as fruit matures an inhibitor of ethylene may be decreasing allowing the fruit to be more responsive or sensitive to ethylene or that an effector is needed for ethylene action (93).

Apparently a threshold level of ethylene is required for a response at a green stage of plant tissue development. The threshold level for ethylene action decreased from 10 to 0.1-1ppm as apples matured. Propylene could stimulate ethylene in 'Red Delicious' apples at all stages of maturity (207); however, a shorter lag time was observed with mature fruits which shows an increase in sensitivity (207). Until apple tissue is at a stage advanced enough to be responsive to ethylene, there is no effect of applied ethylene (93). Pre-climacteric 'd'Anjou' pears ripen after 12-48 hours exposure to 500 ppm ethylene and ripened with the 48 hour treatment even though there was no respiratory  $\text{CO}_2$  climacteric (88). This implies that the respiratory  $\text{CO}_2$  climacteric is an effect of and not a cause of ripening in pears. Tomatoes show a somewhat different situation. Young, green tomatoes have a respiratory climacteric, but no ethylene production or ripening occurs. A respiratory climacteric and ethylene production occur during ripening of older tomatoes (214). Pre-climacteric 'd'Anjou' and 'Packham's Triumph' pears soften in response to 500 ppm ethylene at 1%  $\text{O}_2$  and 10%  $\text{CO}_2$  (35). Added

ethylene had little effect on firmness, soluble polyuronide and peel chlorophyll in apples (124). Loss of galactose residues from the cell wall was similar at applications of high or low ethylene concentration (124).

Levels of ACC and ethylene have been shown to correlate closely for tomato, avocado, and banana during ripening (106).

An inhibitor, daminozide, suppressed ethylene levels in pears and it appears that the effect is on ripening rather than on other aspects of fruit maturation (148).

#### Cold Requirements for Ethylene Production and Ripening

Winter pears have a cold or chilling requirement before ethylene production begins and ripening can take place. Storage at  $-1^{\circ}\text{C}$  abolished the lag in ripening of 'Conference' pears (123). Short cold treatments to 'Bartlett' pears increased ethylene and hastened its production (240), thus promoting ripening (148). 'Bosc' pears ripened better with higher ethylene production if held at  $5^{\circ}$  for 6 days before being ripened at  $20^{\circ}$  (208). However, the best ripening for 'Bosc' pears required 20 days or less at  $-1.1^{\circ}$  and 20 ppm ethylene production (48,50). 'd'Anjou' pears require approximately 50 days at  $-1.1^{\circ}$  (48,50,184). 'Golden Delicious' apples show an analogous response to 'Bartlett', 'Bosc', and 'd'Anjou' pears in that evolved ethylene is promoted by low temperature exposure (126).

The effect of cool temperatures on ripening and ethylene production can be observed on the tree as well as in cold storage as evidenced by premature ripening in 'Bartlett' pears (148,240). Cool temperatures in ~~essence~~ <sup>本質</sup> caused ripening on the tree of 'Bartlett', as shown by increased ethylene production and climacteric respiration (240). Only 3 days of cooling caused noticeable acceleration of maturity in 'Bartlett', so cool temperatures on the tree can affect storage life. Exposure to 18° days and 8° C nights caused early acceleration of ethylene production and climacteric respiration in 'Bartlett' pears whereas 24° day and 16° nights did not (240). During premature ripening in 'Bartlett' pears, soluble pectin, firmness, protein nitrogen, and organic acids were comparable to those in ripening fruit (240). Daminozide delayed 'Bartlett' pear ripening, but cold exposure could reverse this (148); therefore, daminozide must influence endogenous ripening mechanisms. Premature ripening caused by cold nights in 'Bartlett' pears could be controlled by gibberellins or daminozide (240), implicating interactions of plant hormones besides ethylene.

Before the cold requirement is complete in 'd'Anjou' exogenous ethylene application can induce ripening and softening (235). Maturity and time in storage of 'd'Anjou' pears will effect their response to the ethylene application (241).

The steps in ethylene biosynthesis where cold temperature affects the synthesis has been briefly examined in fruit. Softening lagged behind the autocatalytic ethylene production

stimulated by cold in 'Bosc' pears (208). Only after cold exposure stimulates ethylene production, do other ripening changes, such as softening, take place. ACC synthesis increases with chilling injury of cucumbers, however the conversion of ACC to ethylene is inhibited by cold temperatures (233,234). ACC synthase activity increases only on warming of chilled cucumbers and this increase can be inhibited by cycloheximide (234).

### CONCLUSIONS

Ethylene is vital to the proper ripening of pears and can initiate senescent changes in fruit. The surrounding gas atmosphere in storage can influence the ethylene production by fruit, and other plant constituents such as phenolic acids. Environmental and chemical promoters and inhibitors of ethylene production are important aids in prolonging storage life of fruit and understanding the mechanisms of fruit ripening and ageing. The physiological and biochemical changes during the storage life of a commodity influence the ultimate quality and marketability.

## PHENOLIC ACIDS IN PLANTS

### Introduction

The presence of phenolic acids in plants have been known for years because of their participation in the browning reaction in plants. However, it is clear that they also have other roles in plant physiology. Plant phenolic acids, their composition and occurrence, and their metabolic functions have been reviewed (72,186,194,212,249).

Phenolic acids can occur as simple phenolic acids, such as caffeic acid or as esters such as p-coumaroyl quinate or chlorogenic acid. Phenolic acids can also be linked to other compounds by glycosidic linkages. More complex phenolics such as flavonols (eg. catechin), coumarins (eg. umbelliferone), or anthocyanins (eg. cyanidin) are also common in plants. Large molecular weight polymerized products composed of individual phenolic acids are referred to as tannins.

Phenolic acids have roles in enzyme regulation (230), fruit browning (102), fruit astringency (84), cell wall synthesis particularly lignification (39), disease resistance (168), allelochemic effects (169), and effects on plant growth and development (72).

Plant enzymes are affected by phenolics because of the ability of phenolics to bind protein. Knowledge of the phenolic composition of a plant aids in enzyme isolation and plant organelle extractions (230). The role of phenolics in in situ



enzyme regulation is, at present, not well characterized and largely speculative, although their role in IAA oxidase (138,154,213) and peroxidase (108,150) has been partially characterized.

The flavonols such as catechin, can be the substrates for oxidative browning or act as anti-oxidants and protect compounds such as ascorbic acid from auto-oxidation (102). Loss of astringency in fruit is due to the polymerization of simple phenolics into more complex tannins (24).

木质, 木质素  
Lignin, one of the basic components of plant cell walls, is composed of phenolics such as p-coumaric acid and coniferyl alcohol bound to other cell wall polysaccharides (39).

Individual phenolic acids and their oxidation products, have been extensively studied for their role in fungal and bacterial disease resistance in plants (168). It has been hypothesized that the chlorogenic acids and related compounds originated as regulators of plant metabolic processes then acquired allelochemic properties (168). Phenolics also function in soil chemistry (83).

Phenolic acids have been increasingly implicated in growth and development processes. Indoleacetic acid (IAA) oxidase (138,154,213) and peroxidase (108,150) can be inhibited or promoted by phenolics depending on the individual phenolic acid. Phenolics have been shown to be active in rooting (61,180,199), seed germination and dormancy (22,69,173), abscission (21), respiration (60,127), and possibly fruit ripening (75,243).

From the numerous and diverse effects that phenolics can have on plants, it is hardly likely that phenolics are simply metabolic waste products as some writers have suggested. They can clearly influence plant physiological and developmental processes and therefore, examination of plant phenolic composition and quantities present is information basic to the further understanding of plant functions.

#### Methods of Analysis for Phenolic Acids

Methods of analysis for phenolic acids have improved considerably over recent years. Total phenol methods (219) have been used for years , however, the lack of specificity is an extreme drawback.

Paper chromatography, especially two-dimensional systems, have been used for phenolic acid analysis and work well. The limitation of paper chromatography is that quantification is difficult. Many methods, solvent systems and identification methods for paper chromatography have been developed (45,104,194). Thin-layer chromatography methods have also been used for phenolic acid analysis (225). Conversion of trans-cis isomers in relation to light and pH have been examined (119).

High-performance liquid chromatography (HPLC) has more recently made it possible to identify and quantify phenolic acids. Methods using reverse-phase columns and methanol:acidified water solvent systems have been developed for a variety of plant materials and situations (57,92,96,177,187,188,217,226,232,254).

The number of HPLC methods stems from the fact that in a given plant extract it is difficult to separate all compounds of interest by a single method (177). A plant extraction method for HPLC analysis has been developed (175).

A comparison of methods for analysis of sweet potato phenolics has been done (231) using an HPLC method as the standard.

#### Phenolic Acids in Pome Fruits

Phenolic acid composition in the genus Pyrus has been studied (45,46,47) and some rough attempts at quantifying the phenolics present have been made. Total polyphenols and lignin content in cell walls were parallel over the growing season for pears (198). However the total phenolic method did not give information on the individual phenolics and it was found that the composition of pear tannin complex in pears changed with maturity (198).

Extensive paper chromatographic studies of the composition of pear leaf phenolics in different species of Pyrus and their hybrids have been made (44,45,46,47,99). In addition to some of the more common phenolic acids, arbutin has been found in pears. Arbutin is a combination of hydroquinone and glucose, and is not found in most plants. Arbutin has been found in chloroplasts of pear leaves, but not in chromoplasts, leucoplasts or the cytoplasm (99). Plastids in roots and hypocotyls of cotton seedlings are

intimately involved in phenolic metabolism (171). p-Coumaroyl-arbutin and caffeoylarbutin were also found in Pyrus leaves (47).

Studies of phenolic acids present in fruit have been difficult in the past due to the low amounts of phenolics present in fruit as compared to leaves. Epicatechin, catechin, chlorogenic acids, possibly arbutin, quercetin and two unidentified compounds were reported earlier in pear fruit (189). Chlorogenic acid concentrations were greatest, followed by catechins and quercetin. Flavonol glycosides in 'Packham's Triumph', 'Bartlett', 'Bosc' and 'd'Anjou' pears have been identified and compared (67). 'd'Anjou' pears were most different in phenolic composition compared to the other varieties. In an earlier study (251), the distribution and composition of phenolics in apple and pear trees was done. All that was found in fruit was chlorogenic acid, leuco-anthocyanin complexes, and arbutin in seeds. When an attempt was made to quantify the amounts of individual phenolic acids present in pears during ripening it was found that there were increases in chlorogenic acid, catechin, epicatechin, leucoanthocyanins, and unidentified phenolic acids, whereas quercetin levels remained unchanged (189).

Williams (43,250) tentatively identified p-coumaroyl quinate in pears and apples. p-Coumaroyl quinate may be a precursor to chlorogenic acid (140). Both mature and immature apples have the 4-ester of p-coumaroyl quinate as the major isomer (247). Concentrations of p-coumaroyl quinate in apples were found to be

greatest near the skin and least near the core, and that both chlorogenic acid and p-coumaroyl quinate decreased as apples matured (178).

### Phenolics as Inhibitors

Phenolics are usually considered to be natural plant growth inhibitors. Phenolics slow elongation and growth in barley seedlings and may serve as a point where the environment can affect an endogenous chemical to elicit a growth response. This would be true regardless of the environmental stress that induced the increase in phenolics (183).

Tannic acid, gentisic acid, p-coumaric acid, caffeic acid, ferulic acid, p-hydroxybenzoic and syringic acids can all inhibit mung bean hypocotyl growth (60). This role of growth inhibition is apparently dependent on the individual phenolic and plant hormone levels. Abscissic acid inhibits hypocotyl growth; however, a variety of phenolic acids can reverse this, despite the fact they can act as growth inhibitors themselves (190). Cinnamic acid, coumarin, gallic acid, resorcinol, and tannic acid have also been shown to inhibit abscissic acid-induced abscission of beans (21).

Phenolic acids can also inhibit root growth under some conditions and promote it under others. Growth of adventitious roots is generally inhibited by all phenolic compounds (180).

Phenolic acids in soil can inhibit root growth (83), and cause premature vacuolation in meristematic barley root cells.

Phenolics have been shown to affect seed germination and dormancy. p-Coumaric acid is the main phenolic in maple seeds, declines during stratification, and showed inhibition during germination tests (69). Seed coat phenolics from acid hydrolysis decline during stratification of sugar pine seed, but the authors felt there was no correlation between the level of phenolic acids from acid hydrolysis and seed dormancy (173). Sugar pine embryo phenolics subjected to alkaline hydrolysis showed increases in cinnamic, p-coumaric and ferulic acids during stratification, and the authors felt that active synthesis accounted for the increase in phenolics and implied no role for phenolics in dormancy (174). However, the hydrolysis conditions used made it impossible to determine the levels of free and polymerized phenolics and free phenolics are usually the active form. There are increases in the levels of chlorogenic acid and caffeic acid during tobacco seed maturation (202). The free phenolic acids present in Lilium seeds have been shown to inhibit seed germination (22). Dormant buds of sugar maple had ferulic, vanillic, p-coumaric, and caffeic acids, which all showed a 20% growth inhibition in germination tests (222).

### Phenolic Acids and Rooting

Allelopathic effects, where exudates of one plant chemically affects another, have been attributed to phenolics present in the soil (169). In rooting it appears that there is an interaction between auxin levels and promotion of root growth by phenolics. p-Hydroxybenzoic acid, p-coumaric acid, and ferulic acid promoted auxin-induced rooting. Ethylene produced the same stimulatory response as the phenolics (199). Coumarin will stimulate rooting in Impatiens (61), mimicking the effect of auxins.

### Phenolic Acids and Auxin Metabolism

There is a proven relationship between some of the phenolic acids and auxin metabolism. It appears that under some conditions certain phenolics regulate auxins via indoleacetic acid (IAA) oxidase (87,172). Phenolics can promote or inhibit IAA oxidase in pea roots (157). In pea stems, p-coumaric acid activates IAA oxidase in addition to decreasing the inhibitory effect of chlorogenic acid on IAA oxidase (213). p-Coumaric and ferulic acids can decrease bound IAA in maize stems (138). Apparently they inhibited formation of bound IAA but not the activity of IAA oxidase. Caffeic acid and protocatechuic acid had no effect.

The effect of phenolics on abscission and IAA seems unclear at this time. Coumarin and IAA retarded abscission and senescence

in bean abscission zones (5). However, phenolics had no effect on abscission in Coleus either in the presence or absence of IAA (79).

There are two hypotheses on how phenolics affect changes in auxin metabolism. First, phenolics may regulate IAA levels in tomatoes under unfavorable environmental conditions, during which phenolics usually increase (155). Secondly, from experiments with tomatoes the suggestion was made that phenolic acids could act on growth processes via regulation of polar auxin transport. It appeared that phenolic acids delayed transport of auxins (154).

#### Phenolic Acids and Enzyme Regulation

In addition to regulation of auxins through IAA oxidase, phenolics can regulate other enzymes and cofactors that may be involved in plant development and senescence. It has been proposed that polyphenol oxidase, peroxidase, and IAA oxidase are somehow linked and that ethylene can effect the levels or activities of these enzymes (103). The peroxidases have been proposed to have a role in plant and animal senescence. There appears to be a definite relationship between phenolic acids and peroxidases (212). As a general rule phenolic acids which do not form quinones easily are activators or inactive with peroxidases and ortho- and dihydroxy- phenols are inhibitory toward peroxidases (212). Peroxidase is believed to have a role in fruit ripening and senescence. Peroxidase activity in 'Golden Delicious' apples shows two peaks, one at the climacteric, and one



at the start of senescence (86). Hydrogen peroxide or peroxidases may be necessary for ethylene synthesis in plants (82). Peroxidases have been suggested as taking part in the conversion of ACC to ethylene. Chlorogenic acid will inhibit isoperoxidases from tobacco cell and suspension cultures (108). Coniferyl alcohol and ferulic acid have been shown to regulate peroxidases necessary for lignification of cell walls (150). p-Coumaric acid is a very potent activator of the oxidation of NADH by peroxides (212). Phenolic compounds may also regulate  $H_2O_2$  intracellular concentration.  $H_2O_2$  is considered to have a "second messenger" function in rat liver mitochondria and may help metabolic regulation through its regulation on the action of insulin (220). For example, p-coumaric acid will inhibit  $H_2O_2$  production in rat liver.

A possible connection between senescence and phenolic acids is through the quenching of free radicals. Oxy free radicals may be responsible for induction of senescence in plants and animals (139). Benzoic acid can act as a free radical scavenger and many phenolic acids are benzoic acid derivatives, or can act as radical scavengers.

Phenolics may regulate enzymes that control general plant metabolism. Chlorogenic acid can inhibit glucose-6-phosphate dehydrogenase isozymes, thus potentially influencing the activity of the hexose monophosphate pathway (108). In addition to glucose-6-phosphate dehydrogenase, phenolic acids generally tend

to inhibit pyridoxal phosphate requiring enzymes (212). ACC synthase, which converts SAM to ACC in ethylene synthesis, is a pyridoxal phosphate requiring enzyme. Chlorogenic acid can also inhibit methionine decarboxylase thus altering the pathway to polyamine biosynthesis (212). Methionine is one of the major precursors of ethylene. Through regulation of peroxides and peroxidases it is possible that phenolic acids could also regulate ethylene synthesis at the conversion of ACC to ethylene.

#### Phenolic Acids and Ethylene

The evidence for an effect of phenolic acids on ethylene production is, at best, confusing due to many conflicting reports. Monohydroxy phenols, such as p-coumaric acid, were shown to stimulate ethylene production from two model systems, one utilizing cauliflower florets with  $\alpha$ -keto- $\gamma$ -methylthiobutyrate substrate and another involving horseradish peroxidase and methional, and both utilizing peroxidase or hydrogen peroxide (132,153,243,256). However, chlorogenic acid and other dihydroxy phenols were inhibitory to the model systems.

An unhydrolysed extract of ripe tomato skin inhibited ethylene production in green tissue plugs. However, when p-coumaric acid, ferulic acid, or chlorogenic acid were given to tomato tissue plugs there was no effect on ethylene production (243).

Reports of phenolic acids affecting ethylene production have been made. p-Coumaric acid and salicylic acid had no effect on growth of cress root seedlings. However, the halogenated phenolic,

3,5-diiodo-4-hydroxybenzoic acid promoted growth and inhibited ethylene production (195). p-Coumaric acid and catechol in the absence of IAA, but not in the presence of IAA, promoted ethylene production in Coleus blumei explants (79). Coumarin stimulated ethylene in etiolated bean hypocotyl hook opening (170).

Further correlations have been made between ethylene production and phenolic acids, because it appears that ethylene can increase some phenolic acid levels. Ethylene was shown to induce high phenylalanine ammonia-lyase (PAL) activity in lettuce (109). It was also found that ethylene stimulated chlorogenic acid production to a limited extent, along with peroxidase and PAL in sweet potato (115). Russet spotting in lettuce is known to be caused by ethylene, but no correlation was found relative to polyphenol oxidase (PPO) or peroxidase (112). There was an increase in phenolics, chlorogenic acid among them, with development of russet spotting in lettuce (112).

There was a sharp increase in free p-coumaric acid in ethylene treated peach fruit compared to control fruit. Bound p-coumaric acid and abscisic acid did not change. It was hypothesized that p-coumaric acid might play some regulatory role in fruit maturation through effects on IAA oxidase (80). The ethylene inhibitor, aminooxyacetic acid (AOA), also was shown to inhibit PAL activity while increasing the precursor aromatic amino acid pools (105).

### Phenolic Acids and Plant Development and Storage

Changes in phenolic acid composition and quantity have been studied in a variety of horticultural products during maturation and subsequent storage. Total phenolics decrease in banana pulp during storage. Within limits, the higher the temperature the more rapid the loss (23). Total phenolics also decreased in mango during storage (23). Peach fruits with abscission potential contain higher amounts of p-coumaric acid (80). Phenolics decreased in pecans during storage and it was felt that residual phenolics helped to maintain sensory quality and storage stability against rancidity (206).

Total phenolics in plums increase with development (103). However, decreases in catechins and hydroxycinnamic acids have been observed for berries and other fruits during maturation (102). The level of phenolics in carrots increased with time in cold storage but was reduced by CA storage (179). Ethylene was found to induce formation of isocoumarin, eugenin and two other unidentified phenolics in carrots. Isocoumarin was related to bitter taste caused by exposure to ethylene (181).

Tomatoes have been used in several studies investigating the changes in phenolics during maturation and ripening. Tomato fruit wall tissue was found to contain p-coumaric acid, caffeic acid, ferulic acid, and chlorogenic acid (227). There are conflicting reports on the changes that occur during ripening in tomatoes. Walker (227) found that during ripening of tomato, caffeic acid,

chlorogenic acid and ferulic acid increased. Fleuriet and Macheix (75) found that in tomatoes chlorogenic acid decreased over development and ripening. m- and p- Coumaric acids have been found in tomato cuticle and they increased during fruit development (111). p-Coumaroyl quinate acid and feruloyl quinate appeared with the onset of ripening (75). Chlorogenic acid decreases during maturation of tomatoes then increases during the climacteric. As tomatoes ripen, hydrolysed extracts show an increase in ferulic, sinapic and p-coumaric acids and other unidentified phenolics (243).

In 'Cox's Orange' and 'Sturmer' apples chlorogenic acid and p-coumaroyl quinate fell during early maturation to a steady level at maturity (228).

In 'd'Anjou' pears chlorogenic acid and total phenols decreased during development to maturity but increased during storage (237). 'd'Anjou' pears growing in the sun had more phenolics than shaded fruit (236). Also 'd'Anjou' pears which had the physiological disorder, cork spot, had more chlorogenic acid and higher ethylene levels (236). Total phenolics and chlorogenic acids in normal 'd'Anjous' increased up to 7 to 9 weeks in cold storage then remained the same for the duration of the storage period (237).

Changes in phenolics during CA storage have been studied in snap beans. An elevated CO<sub>2</sub> atmosphere (20-30%) inhibited an increase in phenolics in snap beans during storage (40,100).

### Phenolic Acids and Respiration

Since pome fruits show a characteristic rise in respiration during ripening, any control that phenolic acids have over respiration might be of potential value in extending the storage life of fruit.

p-Coumaric and ferulic acids were shown to cause a slight reduction in respiration and respiratory control in mung bean mitochondria (127). Cinnamic and benzoic acid derivatives and flavonoids inhibited, by 50%, photosynthesis in isolated spinach chloroplasts and respiration in isolated potato mitochondria (223). Mung bean mitochondrial respiration, substrate-supported  $\text{Ca}^{++}$  and phosphate transport and respiratory control are inhibited by p-coumaric acid, tannic acid, and gentisic acid. Protocatechuic acid had no effect (60). Vanillic acid was shown to selectively inhibit  $\text{Ca}^{++}$  uptake by mung bean mitochondria (60), but did not affect respiratory control.

In sweet potato mitochondria, oxidative phosphorylation is inhibited by catechol and chlorogenic acid. Ascorbic acid can reverse this inhibition. The quinone forms of the phenolics are thought to be the active inhibitory form (143). In another study chlorogenic acid and catechin were found to be inactive, as inhibitors against ATP formation in mitochondria; however, some flavonoids were active (216). Kaempferol was shown to inhibit

corn mitochondrial phosphorylation (128). Batatasins, phenolics present in dormant yam bulbils, inhibit respiration in mitochondria (113).

Catechol and hydroquinone derivatives can inhibit the beef heart mitochondrial succinoxidase enzyme system (51).

#### Phenolic Acids and Cell Membranes and Walls

Fruit softening during ripening and storage is caused by changes in the cell walls. Phenolic acids are among the building blocks for lignified cell walls. Degradability of corn cell walls by cellulase is highly negatively correlated with amounts of phenolics present in the cell wall. Ferulic and p-coumaric acids were the major phenolics (97).

Several studies have been done on the role of phenolic acids in cell wall development in grasses. Bound ferulic acid has been shown to be present in cell walls of the Gramineae (94). Non-mesophyll cells had more p-coumaric acid than mesophyll cells. Both had ferulic acid bound to polysaccharides (95). Ferulic acid is thought to crosslink polysaccharides in wheat seeds and reduce solubility (156). Ferulic-carbohydrate complexes in cell walls render the cell walls of wheat seeds relatively resistant to endogenous hydrolases (78). A phenolic-carbohydrate complex is released from wheat cell walls by cellulase, and this is probably composed partially of ferulic acid (248). Peroxidases in outer epidermal walls of wheat roots may be involved in turnover of phenolic acids crosslinked to polysaccharides (211).

Leuco-anthocyanins showed complete inhibition of apple pectin methylesterase and a substantial inhibition of yeast polygalacturonase. Chlorogenic acid and catechin gave a 30-40% reduction in apple pectin methylesterase activity (182). Some tannins inhibit hydrolysis of low-methoxy pectin, but not the hydrolysis of high-methoxy pectin which is consistent with the manner in which polygalacturonases function in fruit ripening (98).

The inhibition of ion uptake by phenolics is probably caused by a direct effect on the cell membrane. Subsequent removal of phenolics causes immediate recovery and cytoplasmic recovery would take longer. Inhibition of uptake of some ions by phenolics is caused by generalized increase in membrane permeability to other inorganic ions (82). A possible explanation for this phenolic response in some plants is that other plants secrete phenolics in an allelopathic manner and this alteration in ion uptake may be a poisoning mechanism. Depolarization of membrane potentials can be achieved with several cinnamic and benzoic acid derivatives. While the depolarization by cinnamic acids is stronger, there is a strong correlation between the benzoic acids, their lipid solubility and their inhibitory effect on ion uptake (82).

When phenolics are given to roots, one of the first effects is the increased membrane permeability to cations and anions (82). Certain phenolics inhibit phosphate uptake by roots (81) and potassium absorption (82).



### Phenolic Acids and Injury

Changes in phenolic acids in plants have been studied in relation to wounding, disease resistance and browning reactions. During chill-injury of eggplant, high levels of o-diphenols contributed to browning and pitting (1). Chlorogenic acid levels decreased in chill injured eggplants as browning took place (131). Higher polyphenol oxidase activity also corresponds with storage injury. Phenolics increased two fold in injured orange peel (115). The authors felt that many of these phenolics were for lignin formation to provide a barrier to disease.

Discoloration of snap beans after injury was associated with an increase in phenolics rather than increased activities of phenolase or peroxidase activities (100).

Qualitative and quantitative changes in phenolics occur with injury to tomatoes (74). Near injured zones, p-coumaric derivatives appeared and there was an increase in sinapic acid derivatives. Chlorogenic acid decreased for the first three days then increased to higher than the original level. The authors felt that increases in phenolics might influence the early ripening of injured fruit (74), although wound ethylene might be expected to play a major role.

Total phenolics and chlorogenic acid increased up to 7-9 weeks in stored pears then leveled out; however, susceptibility to friction discoloration continued to worsen up to 17 weeks in

storage (237). Frictional discoloration of pears was reported to be associated with chlorogenic acid levels rather than total phenol content (134).

#### Phenolic Acids and Disease

Phenolic acids have been reported (229) to increase as a result of disease in fruit and may influence disease development. Rotted apple tissue infected by Penicillium expansum had increased amounts of caffeic acid, p-coumaric acid, and ferulic acid. Some evidence suggested that p-coumaric acid and ferulic acid inhibit oxidation of catechin and chlorogenic acid. The existence of p-coumaric and ferulic acids were thought to be the result of fungal hydrolytic enzymes acting on fruit cell walls (229).

Apple pulp infected by Aspergillus niger stimulated a rise in caffeic acid which appeared after infection. Total phenols as well as leucoanthocyanin, p-coumaroyl quinate acid, and catechin decreased after infection. Total phenols decreased. Chlorogenic acid increased then decreased, or gradually decreased depending on the apple variety. Catechin increased in the first 24 hours then it decreased. Total phenols were higher in healthy tissue with rotting pulp having much lower total phenols (14).

Rotting of apples by Sclerotinia fructigena there was a disappearance of catechins, a large loss of some leucoanthocyanins and chlorogenic acid and the appearance of several new phenolics (55). Chlorogenic acid and catechin, before or after oxidation, were not inhibitory to fungal growth. It was concluded that the

oxidation of the apple leucoanthocyanins inhibited fungal polygalacturonase. Pectin methylesterase activity of S. fructigena was not inhibited by apple phenolics, whereas the fungal polygalacturonase was greatly inhibited (55). Rots of a number of fungi reduced the levels of the individual phenolics, probably due to polymerization (55,56).

Penicillium expansum infection in apples suppresses phenolase and causes the accumulation of caffeic, p-coumaric and ferulic acids (229). Chlorogenic acid and p-coumaroyl quinate will inhibit Botrytis sp. in apples (178).

Browning, whether it be from injury, disease or natural deterioration, has been of interest for years as it obviously reduces the commercial value of plant products. It has been found that naturally occurring enzyme inhibitors and enzyme activity determine the amount of browning in Bramley's seedling apples rather than the total phenolics present (246).

p-Coumaric acid, protocatechuic acid, and p-cresol inhibited polyphenol oxidase browning in avocados when catechol was the substrate. However phloroglucinol, orcinol and resorcinol increased polyphenol oxidase activity (118).

Storage of mushrooms in zero oxygen reduced browning; however, storage in high CO<sub>2</sub> was not highly successful (176). Browning reduced total phenols in apples (56).

Phenolic acids can change the taste of fruit through the effect of their polymerization products (tannins) on astringency.

Phenolics as tannins make an important contribution to mango flavor because of their astringency (23). CO<sub>2</sub> treatments are used to remove astringency from persimmon fruits (162). Soluble tannins decreased, as insoluble tannin increased (31). CO<sub>2</sub> could remove astringency without causing softening so the processes at least in persimmons are separate (31). Apple trees that were adequately fertilized produced fruit that was less bitter and astringent than unfertilized trees. This was related to a 17% decrease in fruit phenolics in the fertilized trees (137).

#### Non-phenolic Inhibitors to Fungal Polygalacturonase

In addition to phenolics, other inhibitors to fungal polygalacturonase are present in plants. Water-soluble extracts of leaves of a wide variety of plants were screened for their ability to inhibit fungal pectinase and cellulase (30). Inhibitors of Monilinia sp. polygalacturonase were found in peach and plum. The inhibitor was macromolecular, and heat-labile (71). Aspergillus niger polygalacturonase can be inhibited by a protein in red kidney beans (73). Non-infected apples, tissue extract can inhibit Sclerotinia fructigena (57). Fungal polygalacturonases can be inhibited by bean hypocotyl extracts. The plant protein appears to resemble phytohemagglutinins or plant glycoproteins that agglutinate animal cells (16).

Weurman (244) found two pectinase inhibitors in pears, one present in young fruit and is thermostable, the other is thermolabile and appears when fruit is three months old and reaches a

maximum in ripe fruit. When active or heat deactivated pectinase inhibitor from pears was added to pectic acid and pear extract no difference in active or heat deactivated could be found, implying that a maximum inhibition was already present in the extract or that the inhibitor doesn't affect the pectinase activity (245). It has been found that the inhibitor doesn't effect endogenous pear polygalacturonase (7). Two inhibitors of fungal polygalacturonase were found in 'Bartlett' pears (6). As 'Bartlett' pears matured suseptibility to Penicillium expansum, Botrytis cinerea, and Dothiorella gregaria increased and a fruit inhibitor to fungal polygalacturonase declined as fruit matured (8). Addition of isolated pectinase inhibitor to pectinase in pears only causes inhibition up to a certain maximum (244). During 'Bartlett' fruit ripening an increasing amount of the inhibitor was extracted in the soluble fraction (8), possibly indicating that the inhibitor is cell wall bound. The inhibitors in 'Bartlett' are proteins , with a molecular weight of 91,000 daltons, composed of two identical subunits, and heat-labile (6). A cup-plate assay method (34,63,64,65,192,193) has been used to study the pear inhibitor to fungal polygalacturonase.

## CHAPTER III.

TITLE: Changes in phenolic acids and internal ethylene during long-term cold storage of 'd'Anjou' and 'Bosc' pears.

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Abstract. Mature 'Beurre d'Anjou' and 'Beurre Bosc' pears (Pyrus communis L.) contained as the major fruit flesh phenolics at harvest: 50 ug/g fresh weight chlorogenic acid, 13 ug/g catechin, 10 ug/g arbutin and no epicatechin or p-coumaroyl quinate for 'd'Anjou' and for 'Bosc' 50 ug/g chlorogenic acid, 14 ug/g catechin, and 19ug/g arbutin and no epicatechin or p-coumaroyl quinate. During 170 days storage of -1° C storage, chlorogenic acid fluctuated but rose and leveled to 70 ug/g in 'd'Anjou' but fell to 30 ug/g in 'Bosc'. Catechin rose to 30 ug/g in 'd'Anjou' and to 20 ug/g in 'Bosc'. Arbutin remained nearly constant at 10ug/g in 'd'Anjou' and 18 ug/g in 'Bosc'. Coincident with the completion of the cold requirement for initiation of ripening and endogenous ethylene production there was an appearance of about 1 ug/g p-coumaric acid derivative at 20 days for 'Bosc' and 50 days for 'd'Anjou'. Later at 120 days there was a dramatic rise to 18 ug/g of epicatechin/p-coumaroyl quinate in 'd'Anjou' but not in

'Bosc'. Exogenously applied phenolics to tissue plugs did not stimulate ethylene production. Bruising the pear fruit caused a 30% increase in chlorogenic acid and a 50% increase in catechin.

## INTRODUCTION

Phenolic acids have been shown to have roles in abscission (1), disease resistance (19), tissue browning (13), enzyme regulation (14,28), and auxin metabolism (16,18,29) in plants. It has been hypothesized that phenolic acids may be involved in fruit ripening (9,10), and ethylene production (11,20,34). On a commercial level the development of browning and susceptibility to bruising are among factors which limit the storage life of pears (7,32). While total polyphenols were found to decrease during development of oriental pears (25), few studies have characterized the changes in individual phenolics during the later stages of pear maturation. By paper chromatography pear flesh has generally been found to contain chlorogenic acid, catechin, epicatechin, and possibly arbutin and p-coumaroyl quinate, along with quercitin and flavonol glycosides (3,4,8,23). The advent of high-performance liquid chromatography (HPLC) has made it possible to examine levels of individual phenolics over time in storage rather than being limited by non-specific total phenolic methods or semi-quantitative methods such as paper chromatography. Our objectives were to determine which phenolics were present in pear flesh, the changes that occurred in phenolics over long-term cold storage, and if any of the individual phenolics acids appeared to be of physiological or regulatory significance.



## MATERIALS AND METHODS

Fruits were harvested from the north side of trees at the Mid-Columbia Experiment Station, Hood River, Oregon at 66.7 N firmness and held at  $-1.1^{\circ}$  C and analyzed upon removal from cold storage. Fruit ethylene production was monitored by gas chromatography. Composite 10 g (FW) samples of pear flesh taken from 3 pears were extracted in hot 2% acetic acid then centrifuged at  $10,000 \times g$  for 20 min. at  $4^{\circ}$  (21). Supernatant samples were then frozen, lyophilized, and redissolved in methanol:acetic acid (98:2). Duplicate samples were analyzed by gradient HPLC. Solvent A was methanol:acetic acid (98:2) and Solvent B was water:acetic acid (98:2). Samples were analyzed on a Beckman HPLC with an Alltech 10 micron C18 (25 cm x 4.6 mm I.D.) reverse-phase column at  $50^{\circ}$  over 45 min in a gradient of 10% Solvent A to 25% Solvent A, then isocratically at 25% Solvent A for 5 min., with the remainder of the mobile phase being Solvent B. UV Detection was at 280 nm. The system was a modification of the systems of Qureshi (22) and Villeneuve (31). Peaks were verified by retention time of sample peaks with known standards: arbutin, chlorogenic acid, p-coumaric acid, and epicatechin from Sigma, St. Louis, Missouri; catechin from Nutritional Biochemical Corp., Cleveland, Ohio; p-coumaroyl quinate was graciously supplied by Dr. Vernon Singleton, UC Davis, Calif. Table 1 gives the retention times of known standards and compounds identified as such in pear flesh. Further confirmation was obtained by two-dimensional paper

chromatography (4) in secondary butanol:water: acetic acid (70:28:2) and water:acetic acid (98:2) and characterized with UV light, ammonia vapor, diazotized p-nitroaniline before and after a spray of 15 %  $\text{Na}_2\text{CO}_3$  in water. In some cases, to further identify certain compounds, the spots were eluted from preparatory paper chromatography then alkaline hydrolysis was carried out at room temperature in 2N NaOH for 4 h under nitrogen and the products were re-acidified before analysis. Absorption spectra in 95% ethanol were compared to known standards.

Pear discs, cut with a No. 6 cork borer (I.D. 1.1 cm) and trimmed to 5 mm thick, were vacuum-infiltrated with 0.35 M mannitol,  $5 \times 10^{-4}\text{M}$  phenolic acid, and/or  $5 \times 10^{-4}\text{M}$  l-aminocyclopropane-1-carboxylic acid (ACC), then held in a rubber serum capped vial. Ethylene was determined by gas chromatography on an alumina column (26).

For the effects of bruising on phenolics, pears after 120 days cold storage were dropped from a height of 20 cm onto a concrete surface.

## RESULTS AND DISCUSSION

Chlorogenic acid was found to be the major phenolic acid (40-70 ug/g F.W.) present in 'd'Anjou' pears (Fig. III.1). During cold storage chlorogenic acid levels initially fluctuated then increase slightly by 170 days. Wang and Mellenthin (32) reported similar increases in chlorogenic acid and noted that browning continued to worsen in cold storage. Catechin, the second most abundant phenolic acid in 'd'Anjou' pears also increases over time in cold storage. The levels of catechin approximately double (from 13 ug/g to 29 ug/g F.W.) over 170 days storage of 'd'Anjou' pears, and it is likely that it is the increase in catechin that might account for the increasing browning capacity (32) of pears after chlorogenic acid levels off. High fruit to fruit variability, gave fluctuations between samplings; however, overall trends in phenolic acid levels are still apparent. Arbutin has been previously suspected in pear fruit (23), but due to the inadequacies of the previous methods it was difficult to detect. The sensitivity of HPLC has made it possible to detect arbutin in fruit. Arbutin levels in 'd'Anjou' pears remain virtually unchanged at 10 ug/g F.W. during cold storage.

'Bosc' pears contain the same major phenolic acids as 'd'Anjou' pears; however, the amounts of the individual phenolics differ (Fig. III.1, III.2, and III.3). Chlorogenic acid is lower in 'Bosc' pears than in 'd'Anjou' and shows no definite pattern or increase. Catechin in 'Bosc' slightly increase over cold storage,

while arbutin remains unchanged. Arbutin is generally higher in 'Bosc' (Fig. III.2) than in 'd'Anjou' pears (Fig. III.1).

A co-eluting peak of epicatechin and with p-coumaroyl quinate and a separate peak of a p-coumaric acid derivative appeared in both pear varieties after time in cold storage (Fig. III.3). In 'd'Anjou' the epicatechin/p-coumaroyl quinate peak appeared after 52 days in cold storage, remained at low levels then increased dramatically after 90 days in cold storage. The large increase at 90 days could have been due to either compound in the fused peak and might signal the onset of a more rapid deterioration of the pear. The same compounds appeared in 'Bosc' pears sometime between 14 and 28 days in storage and remained at low levels for the storage life of the pears. A p-coumaric acid derivative appeared in 'd'Anjou' also at 52 days after cold storage and increased over the remaining storage period. The p-coumaric acid derivative appeared in 'Bosc' between 14 and 28 days and it also continued to increase over cold storage. Due to the small amount of the p-coumaric acid derivative present, further attempts at identification were inconclusive.

Winter pears require a chilling period before proper ripening can take place (5,33) and this cold period seems tied to ethylene levels and sensitivity to ethylene. 'd'Anjou' pears require approximately 50 days in cold storage and 'Bosc' pears approximately 20 days (5,6). The first significant internal ethylene production in 'd'Anjou' was at approximately 50 days approximately 14 days in 'Bosc' (Fig. III.4). The magnitude of

increase in internal ethylene for 'Bosc' is much greater than for 'd'Anjou'. The dates of the start of ethylene production correspond closely with the appearance of the epicatechin/p-coumaroyl quinate and the unidentified p-coumaric acid derivative in both pear varieties (Fig. III.3). The difficulty in detecting low levels of ethylene and phenolic acids and sampling frequency could account for the several day difference in detecting the appearance of both substances. It has been shown that ethylene can increase phenylalanine ammonia-lyase levels (15). Phenolic acids have been implicated in peroxidase reactions in senescence (14,28,30) and can function in free radical reactions which may be involved in ethylene production (2,17,24). The appearance of the epicatechin/p-coumaroyl quinate and p-coumaric acid derivative with the onset of ethylene production suggest that these compounds could be involved in ethylene biosynthesis or closely related senescent changes in fruit. It is interesting to note that p-coumaroyl quinate and feruloyl quinate appeared in tomatoes as they passed from a green to pink color and began to ripen (10). It has been shown that ethylene can increase phenylalanine ammonia-lyase levels (15). Therefore the possibility exists that ethylene stimulated the synthesis of the epicatechin/p-coumaroyl quinate and p-coumaric acid derivative. The appearance of ethylene production and phenolic acids could be coincidental, or signify that changes in the cell wall released phenolic acids (12,27), or that changes in phenolic acid composition are due to

metabolic shifts that occur with the onset of senescence. Fruit firmness declined from 66.7 N (6.8 kg) to 50 N (5 kg) over 170 days in storage (Fig. III.4).

If phenolic acids are involved in ethylene production it does not appear to be a direct effect. *p*-Coumaric acid and catechin at  $5 \times 10^{-4}M$  did not influence ethylene production in d'Anjou pears in the presence or absence of ACC, a precursor to ethylene (Fig. III.5). Arbutin, chlorogenic, and epicatechin also had no effect (not shown in Fig. III.5). Wardale (34) found that exogenous phenolics had no effect on ethylene production in tomatoes.

Since bruising and increased susceptibility to physical injury often limits the storage life of 'd'Anjou' pears, we examined the changes in phenolic acids that occurred with bruising in stored fruit. Catechin and chlorogenic acids levels increased 50% and 30%, respectively, with bruising while arbutin, epicatechin and the *p*-coumaric acid derivatives remained consistent. Both chlorogenic acid and catechin are good substrates for browning reactions and can cause more rapid oxidation of flavonol glycosides (13), thus increasing the browning capacity of the fruit.

## CONCLUSIONS

Increases in chlorogenic acid, catechin, epicatechin and p-coumaric acid derivatives occur with cold storage of 'd'Anjou' pear. Bruising increases catechin and chlorogenic acid levels in pears. The appearance of epicatechin and p-coumaric acid derivatives with the onset of ethylene production and the ability to ripen in pears could have physiological significance. This concept has been suggested in ripening tomatoes where an analagous situation seems to occur (10).

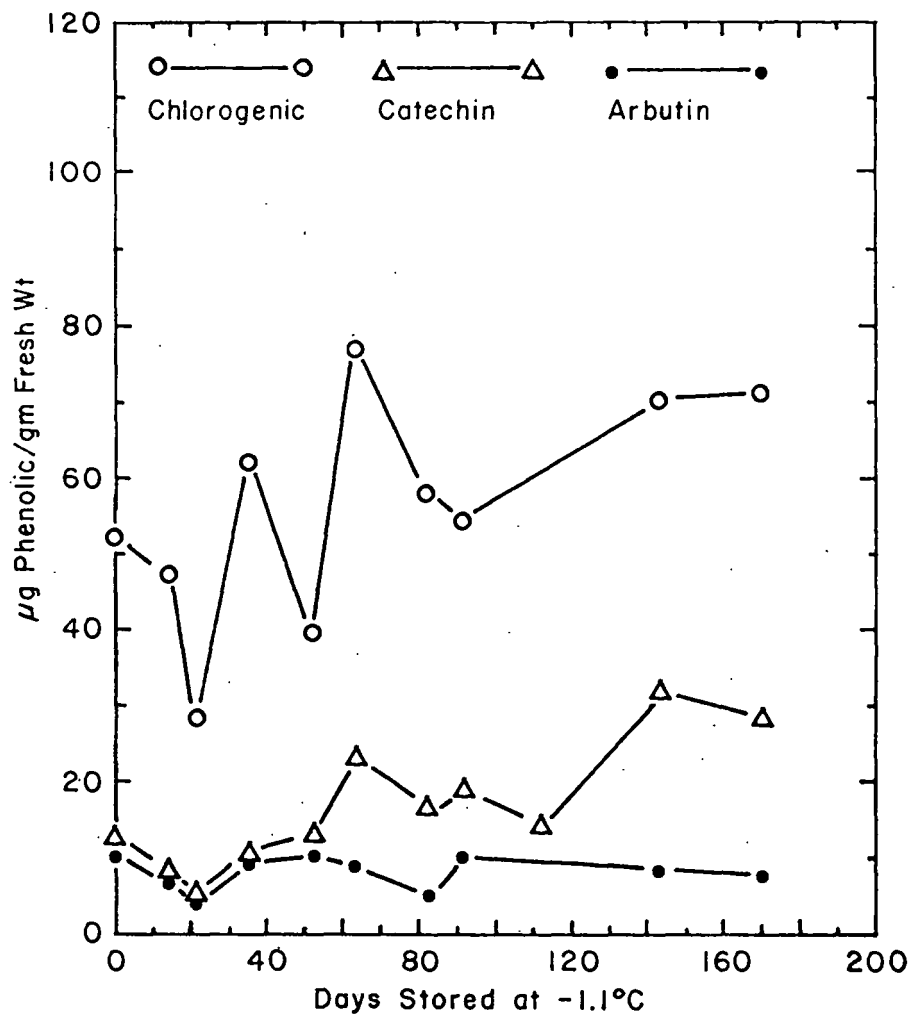


Fig. III.1. Chlorogenic acid, catechin, and arbutin in 'd'Anjou' pear flesh during storage at  $-1.1^{\circ}\text{C}$  for 170 days.



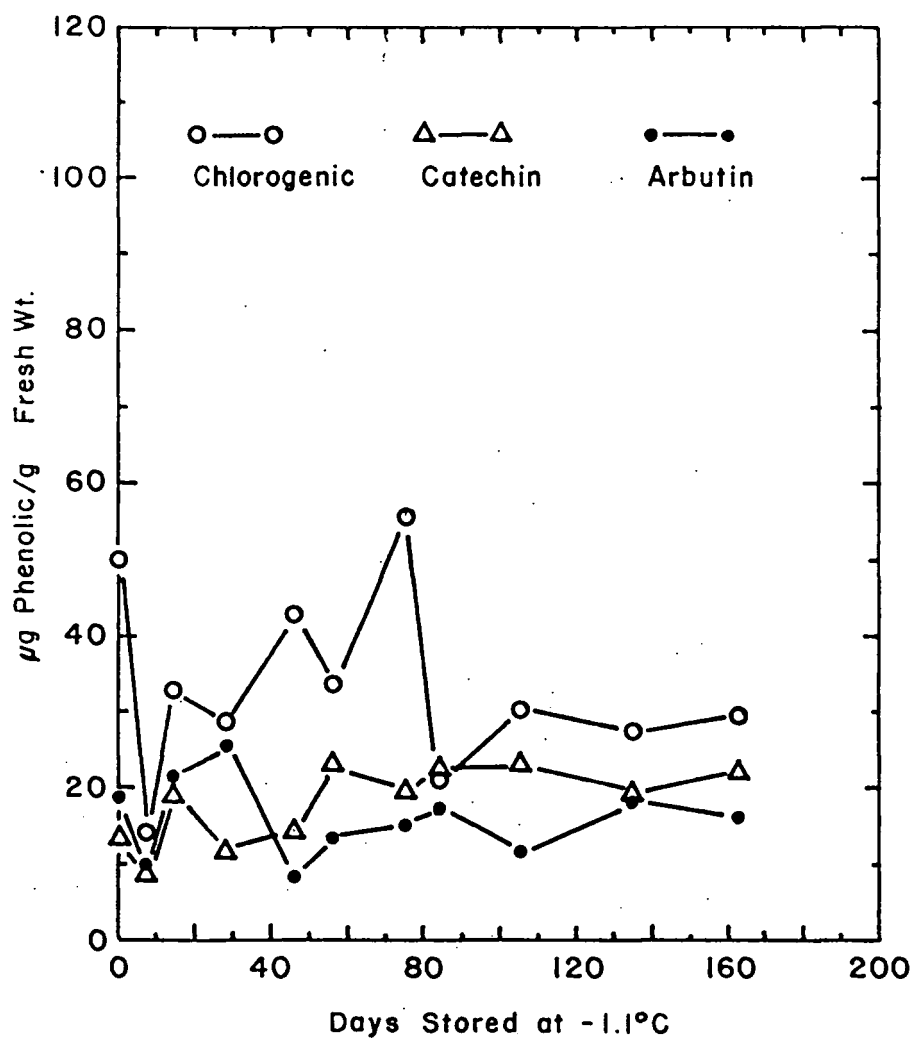


Fig. III.2. Chlorogenic acid, catechin, and arbutin in 'Bosc' pear flesh during storage at  $-1.1^{\circ}\text{C}$  for 160 days.

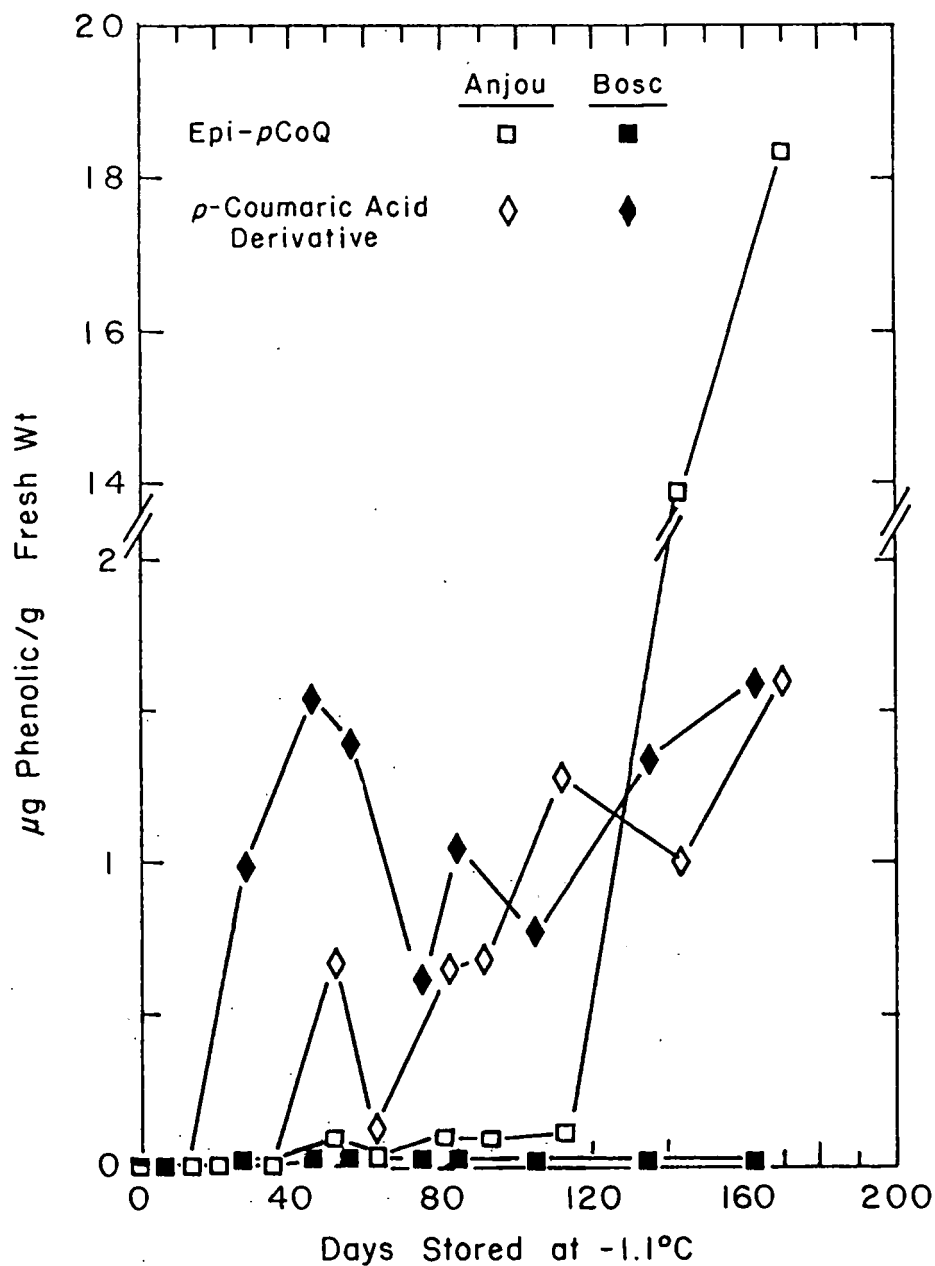


Fig. III.3. Changes in epicatechin/p-coumaroyl quinate (Epi-pCoQ), and a p-coumaric acid derivative in 'd'Anjou' and 'Bosc' pears during storage at  $-1.1^{\circ}\text{C}$  for 160 days.

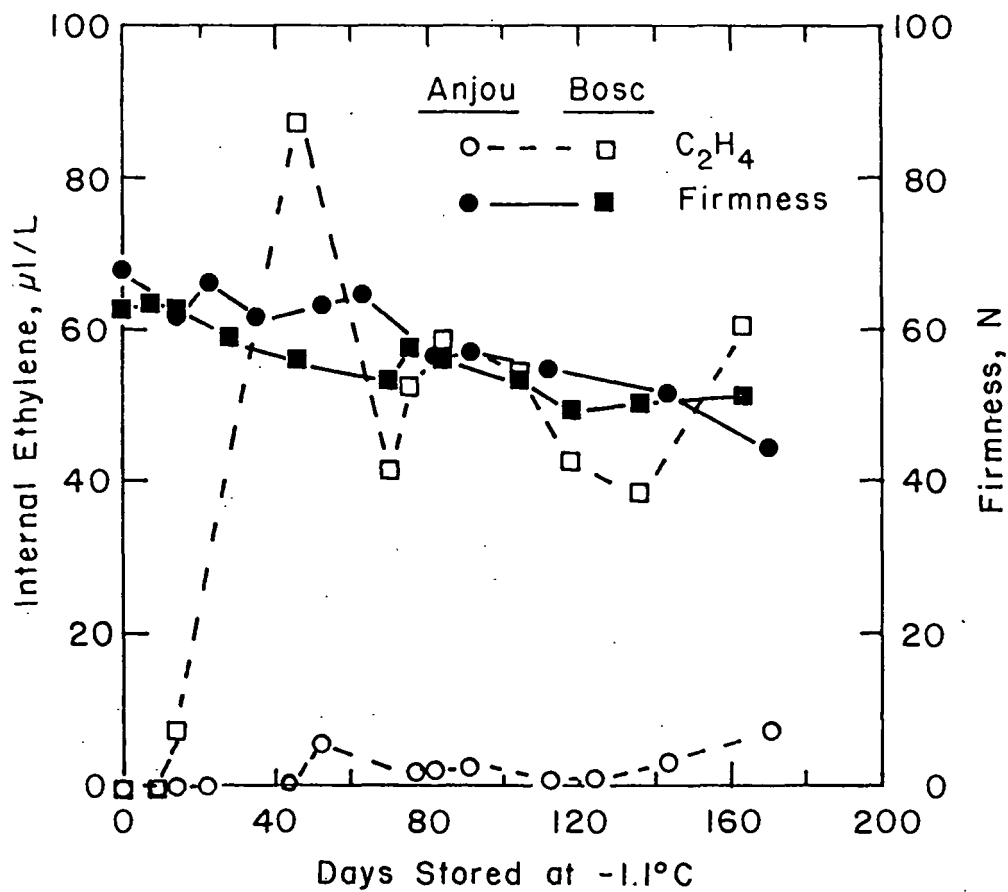


Fig. III.4. Internal ethylene levels and fruit flesh firmness of 'd'Anjou' and 'Bosc' pears for 160 days storage (-1.1°C).

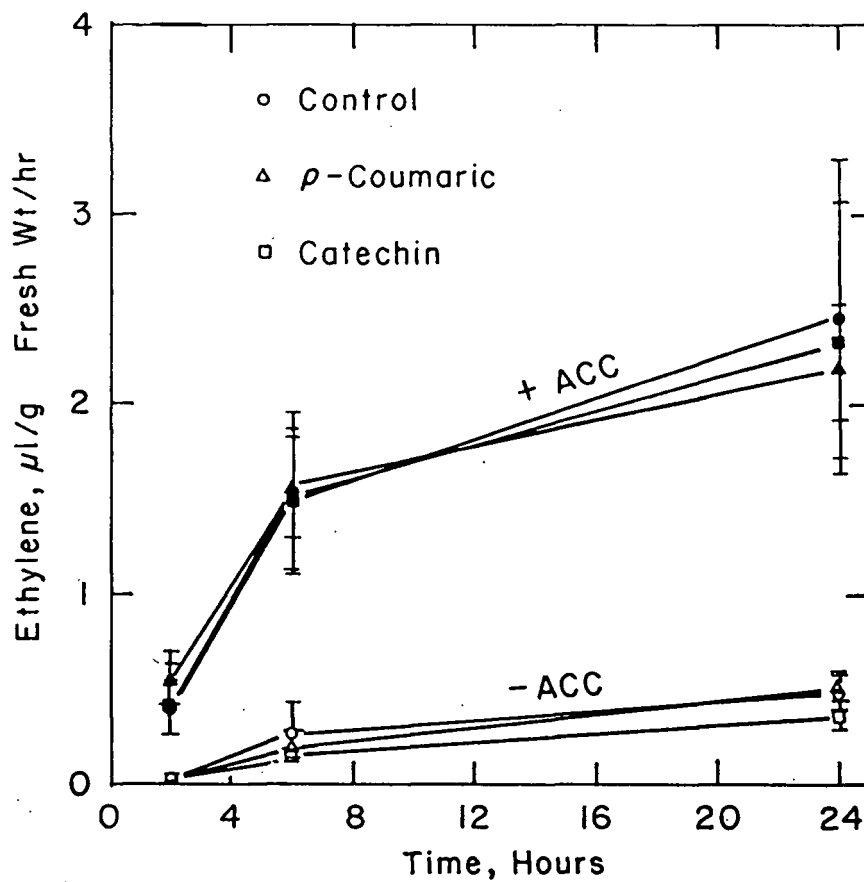


Fig. III.5. Effect of *p*-coumaric acid and catechin on ethylene production from discs of 'd'Anjou' pears in the presence or absence of  $5 \times 10^{-4}\text{M}$  1-aminocyclopropane-1-carboxylic acid (ACC).

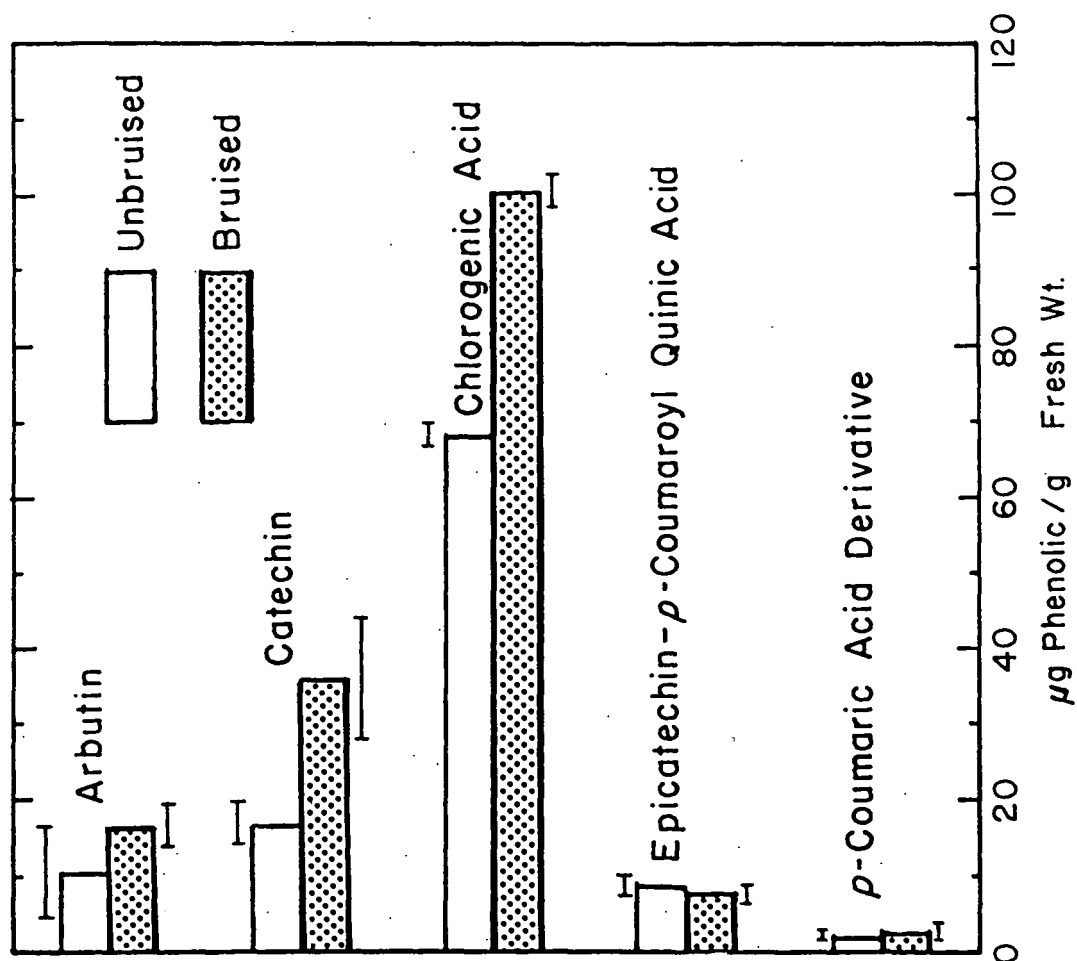


Fig. III.6. Effect of bruising on phenolic acid levels in 'd'Anjou' pear flesh after storage at  $-1.1^{\circ}\text{C}$  for 120 days.

Table III.1. Retention times of standard phenolic acids and compounds from pear flesh extract.

<u>Compound</u>	Retention Times (min)	
	<u>Standard</u>	<u>Pear extract</u>
Arbutin	2.6	2.6
Catechin	12.7	13.1
Chlorogenic Acid	19.9	20.1
Epicatechin	24.0	23.6
p-Coumaroyl quinate	25.1	25.5
p-Coumaric acid	29.2	----

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## CHAPTER IV.

Title: Development of ethylene biosynthesis and ethylene-induced ripening in 'd'Anjou' pears during the cold requirement for ripening.

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Additional index words. Pyrus communis, 1-aminocyclopropane-1-carboxylic acid, quality, ripening, maturity, postharvest physiology.

Abstract. Fifty days in  $-1.1^{\circ}\text{C}$  cold storage were required to stimulate ripening and ethylene synthesis in 'd'Anjou' pears (Pyrus communis L.). Pears at  $20^{\circ}$  were exogenously supplied with 10 or 50 ppm ethylene after 30 days in storage. There was an increase in respiration from 8 mg  $\text{CO}_2/\text{kg/h}$  to 19 mg  $\text{CO}_2/\text{kg/h}$  in ethylene treated fruit over a 12 day period, while fruit held in air showed only a slight increase. Fruit firmness in ethylene treated fruit decreased from 66 N to 10 N over 12 days at  $20^{\circ}$ . Fruit held in air had no significant change in firmness. Extractable juice was unchanged in fruit held in air, but declined sharply in ethylene treated fruit. Taste panel evaluation determined that ethylene-treated fruit was ripe, sweet, and juicy

but fruit held in air was comparable to unripe fruit. Pear flesh discs were unable to convert exogenous 1-aminocyclopropane-1-carboxylic acid to ethylene after 21 days cold storage, but were able to after 41 or 50 days.

## INTRODUCTION

'd'Anjou' pears require approximately 50 days in cold storage at  $-1^{\circ}$  C after harvest to develop the ability to ripen and produce ethylene (5,10). Storage at  $-1^{\circ}$  will abolish the lag in ripening of 'Conference' (6) and 'Bosc' (2,4,11) pears. Cold treatments to 'Bartlett' pears increase ethylene and hasten its production (15), thus promoting ripening (8). The promotive effect of cool temperatures on ripening and ethylene production can be observed on the tree as well as in cold storage as evidenced by the disorder "premature ripening" in 'Bartlett' pears (8,15). Exogenous ethylene application to 'd'Anjou' pears before completion of the cold requirement will induce ripening and softening (13,16). The effect of ethylene on immature (14) and mature 'd'Anjou' pears (16) during fulfillment of the cold requirement has shown that the respiratory  $\text{CO}_2$  climacteric was not necessary for fruit softening to take place and that during maturation metabolic reorganization seemed to be taking place. In chilling injury in cucumber, 1-aminocyclopropane-1-carboxylic acid (ACC) synthesis increases, however the conversion of ACC to ethylene was inhibited (12,13). Cold below  $12^{\circ}$  was shown to inhibit the conversion of ACC to ethylene (1). The system converting ACC to ethylene in apple and tomato has been shown to be sensitive to temperature and is believed to be cell wall-membrane bound (9). Pears, because of their need for a postharvest cold period to induce ripening (5,10) offer an oppor-

tunity to examine the effect of ethylene on ripening characteristics in a mature fruit without the complication of endogenous ethylene and ripening capacity. Our first objective was to determine what changes endogenous ethylene would induce in pears unable to ripen. Our second objective was to investigate changes that occurred during the cold requirement for ripening in 'd'Anjou' pears relative to ACC and the ability to convert ACC to ethylene.

## MATERIALS AND METHODS

Pears were harvested from the Mid-Columbia Experiment Station, Hood River, Oregon, at commercial maturity of 66.7 N (6.8 kg) firmness in September and stored at  $-1.1^{\circ}$  C.

Pears after 30 days cold storage were treated with 0, 10, or 50 ppm ethylene in a flow-through system at 200 ml/min with ethylene metered into the air stream by a capillary system. Ethylene levels were measured in the air stream at the inlet and outlet to each container holding the pears. Ethylene levels were determined by gas chromatography with an alumina column and ACC was extracted in 9% perchloric acid then assayed by conversion to ethylene by the method of Lizada and Yang (13) with 4 replications per treatment. For ACC determination 100 g F.W. of pear flesh was ground in a Wareing blender with 100 ml 9% perchloric acid. The extract was vacuum filtered and brought to 250 ml with distilled water. The equivalent of 10 g F.W. was passed through a Dowex 50W-X4 cation exchange column and ACC eluted from the column with 5 ml of 2 N  $\text{NH}_4\text{OH}$ . The  $\text{NH}_4\text{OH}$  was removed under vacuum. To each 14 ml vial containing the sample, 800  $\mu\text{l}$  distilled water, and 100  $\mu\text{l}$  of 100  $\mu\text{M}$  mercuric chloride were added. The vials were capped with a rubber serum cap and 100  $\mu\text{l}$  5%  $\text{NaOCl}$ :saturated  $\text{NaOH}$  (2:1) was added. The vials were shaken intermittently for 2.5 min. A 1 ml gas sample was withdrawn from the vial and the amount of ACC which had been converted to ethylene was determined by gas chromatography.

Fruit firmness was determined by use of a UC firmness tester with an 8 mm plunger and 2 punches per fruit with 3 replications per treatment. Extractable juice (5) was determined by grinding 100 g pear flesh, cut in sectors from 6 pears, in an Acme juicerator for 1 min. Dampened paper towels lined the juicerator basket to catch the pulp and the liquid portion was defined as extractable juice. Soluble solids were determined with a handheld refractometer. Respiratory CO<sub>2</sub> was measured with an infrared gas analyzer. There were 3 replications per treatment.

Quality evaluation was by 12 semi-trained panelists done on peeled pear sectors and fruit was ranked from 1 = sour flavor to 5 = sweet, 1 = dry to 5 = juicy, 1 = coarse texture to 5 = buttery texture, 1 = unripe to 5 = over-ripe, and overall quality of 1 = very bad to 5 = very good. In September the panel compared unripened fruit, fruit held in air, and fruit treated continuously with 10 or 50 ppm ethylene after all ripening treatments had been at 20° C for 10 days. In November, the panel compared pears ripened in air with fruit treated with 10 ppm ethylene after both were held for 10 days at 20°.

Evaluation of response to added ACC involved discs of pear flesh were cut with a 1.1 cm I.D. cork borer and trimmed to 5 mm thickness. There were 6 discs per 25 ml sealed Erlenmeyer flask, cut from a minimum of 3 pears. There were 5 replications per treatment. Each flask contained 2 ml of solution that was vacuum

infiltrated into discs. ACC concentration was 0.5 mM in 0.35 M mannitol. Control treatments had 2 ml of 0.35 M mannitol. Flasks were aired between each ethylene sample.



## RESULTS AND DISCUSSION

Fig. IV.1 gives the respiration rate of pears in air in September when they do not ripen, and when treated with 10 or 50 ppm ethylene. Included for comparison is the respiration rate of ripening pears in November after the cold period necessary for ripening has been completed, and the pears ripen naturally. After 6 days of ripening at 20° C, ethylene treatment at 10 or 50 ppm increased respiration significantly compared to fruit held in air. Ripening pears in November had overall higher rates of respiration than any of the treatments in September. Even though applications of ethylene increased respiration it has been shown previously that no climacteric rise occurs although other aspects of ripening continue (20). 50 ppm ethylene produced a slightly higher rate of respiration than did 10 ppm ethylene. All fruit in September shows a rise in respiration on removal from storage but the respiration rate of fruit held in air thereafter falls and remains at a low level. This transitory rise in respiration (not to be confused with the climacteric) in the first 24 h out of cold storage has been previously noted in pears (20). The September fruit held in air never softened appreciably while pears treated with 10 or 50 ppm in September or those ripened in November all softened to less than 4.4 N (1 kg) in 12 days (Fig. IV.2). Fruit held in 10 ppm ethylene softened significantly more than fruit held in 50 ppm or fruit ripened in air in November after 9 days of ripening. Softening in 'd'Anjou' was shown to proceed more

rapidly with exposure to 2ppm ethylene than with 1 or 0.5 ppm ethylene (16). Fig. IV.3 shows that extractable juice was significantly lower from ethylene treated fruit compared to fruit held in air in September. Extractable juice is thought to decrease in pears with ripening as more cell sap is bound to a more gel-like cell wall matrix (3). Soluble solids were not significantly different between the treatments (not shown).

To determine which quality characteristics were most influenced by ethylene, taste panel evaluation of pears in September and November was done. Unripened, and air ripened fruit in September could easily be distinguished from fruit held in the presence of 10 or 50 ppm ethylene (Table IV.1). The panel could tell no difference between pears held 10 days in air in September and unripe fruit in September, or unripe fruit in November (not shown), for any of the quality characteristics evaluated. The stage of ripeness and the textural characteristics between coarse and buttery were ranked the same regardless of ethylene levels, despite some measurable differences in fruit firmness (Fig. IV.2). Overall preferences for the treatments were apparently tied to the sweetness and juiciness of the pears. The panel ranked sweetness as no different between 10 or 50 ppm ethylene treated pears in September, or in 10 ppm ethylene treated pears in November. They could, however, distinguish a slight difference in pears ripened in air in November and felt that they were not as sweet compared to pears ripened in 10 ppm ethylene. Internal ethylene in the pears in November after 10 days at 20° C was between 1 and 40 ppm,

and had previously been sufficiently high enough to soften the pears, however it was below 1 ppm for the first 6 days. It appears that continually higher levels of ethylene as in the ethylene treated pears resulted in a sweeter pear.

The juiciness of the pears was judged to be higher in September pears treated with 10 ppm ethylene than with 50 ppm, even though those extractable juice was not statistically different between treatments. November pears ripened in air were judged to be less juicy than pears with 10 ppm ethylene. As with sweetness, it's possible that the continually higher and consistent ethylene levels resulted in this preference.

In evaluating the overall preferences of the panel it appears that they preferred pears held in intermediate levels (10 ppm) of ethylene, when compared to higher ethylene (50 ppm). This preference seems to be related to the dry-juicy component of quality rather than texture or ripeness. High levels of ethylene may produce off flavors, while low levels may not give as sweet a pear or full development of flavor. High levels of ethylene might have also caused the pears to reach optimum quality earlier than at the 10th day when quality was being evaluated by the taste panel.

The ability of 'd'Anjou' pear fruit tissue to produce ethylene in the presence or absence of ACC during the cold storage period necessary to stimulate endogenous ethylene production is shown in Fig. IV.4. When discs are cut from 'd'Anjou' pear after

21 days  $-1^{\circ}$  C cold storage, long before endogenous ethylene production begins, there is eventually a small amount of ethylene production from the discs even though the fruit fails to ripen. Even when given ACC, the discs cut from 21 day stored pears, cannot produce any large amount of ethylene. This would indicate that the enzyme to convert ACC to ethylene or some needed cofactor is virtually missing at this stage or that some endogenous inhibitor is active. A less likely possibility is that ACC is unable to penetrate the tissue at this stage and the cold exposure increases the ability of ACC to penetrate the tissue. After 41 days storage, the control discs were still producing only very small amounts of ethylene by 24 hours. However, if given ACC, the discs were now capable of producing much higher amounts of ethylene. After 50 days of cold storage, ACC is efficiently converted to ethylene. The ability to convert added ACC to ethylene during the later half of the cold storage period suggests that ACC, not the ACC to ethylene conversion system, is the limiting factor in ethylene production in this period. Thus the ability to convert ACC to ethylene appears to be non-functional initially, but then develops prior to ACC synthetase activity in the case of 'd'Anjou' and possibly other winter pear varieties.

## CONCLUSIONS

Exogenous ethylene applied to 'd'Anjou' pear fruit before the completion of the cold requirement promotes softening, a rise in respiration, a decrease in extractable juice and gives acceptable ripened pear quality as judged by a taste panel. Warm temperatures in the absence of added ethylene did little to stimulate any of the changes associated with ripening.

During the first 21 days in cold storage the ability to convert ACC to ethylene develops, or alternatively, permeability to exogenous ACC increases. ACC synthesis then appears to be the limiting step toward the end of the cold period. If fulfillment of the cold requirement could be delayed, then presumably ethylene synthesis would be delayed resulting in a longer storage life of the pear.

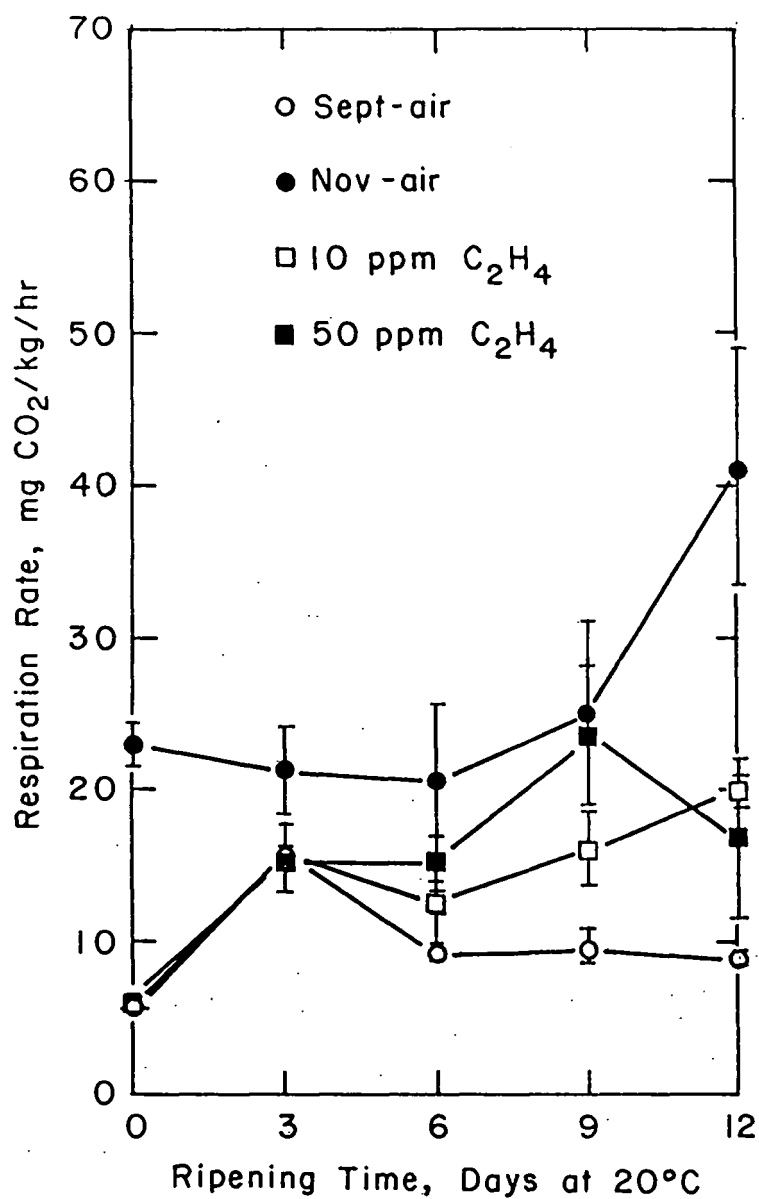


Fig. IV.1. Respiration of 'd'Anjou' pear during ripening in September in air or 10 ppm or 50 ppm ethylene and during November in air.

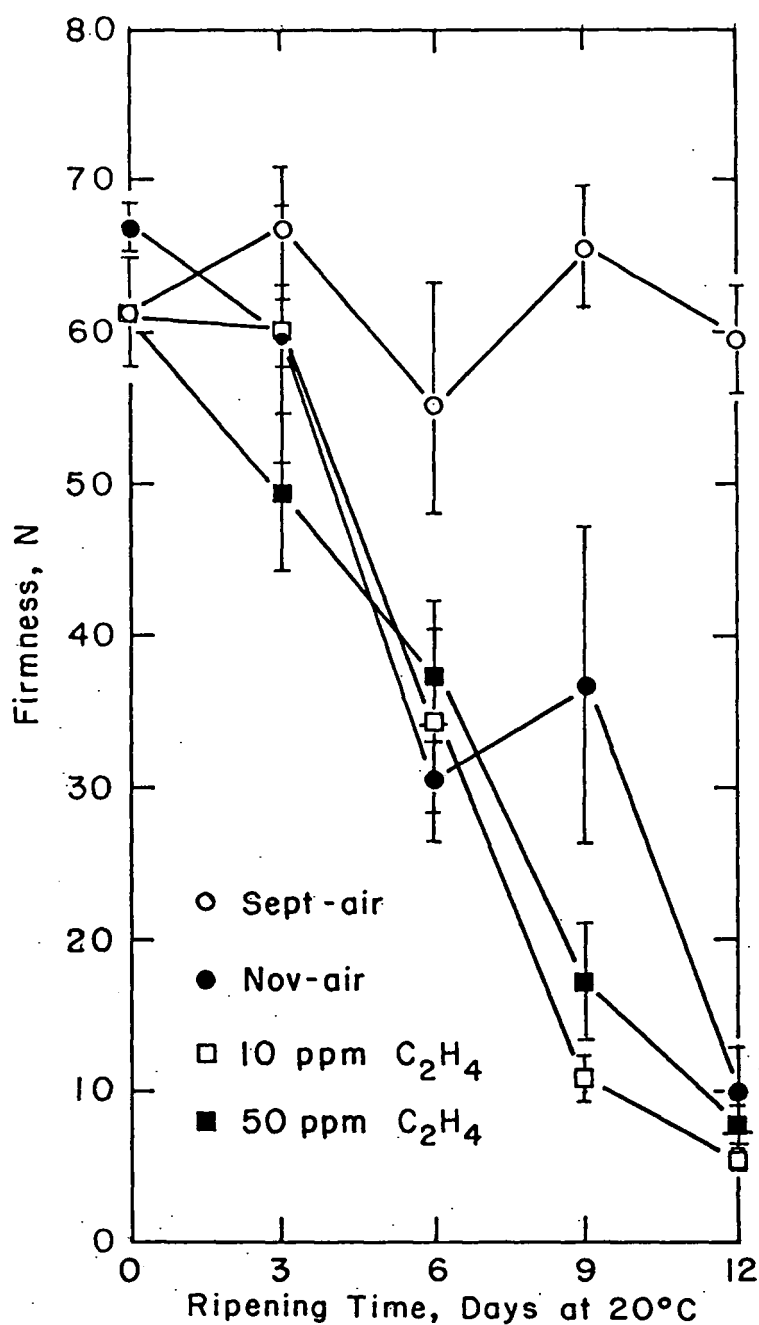


Fig. IV.2. Changes in 'd'Anjou' pear fruit firmness during ripening in September in air or 10 or 50 ppm ethylene and during November in air.

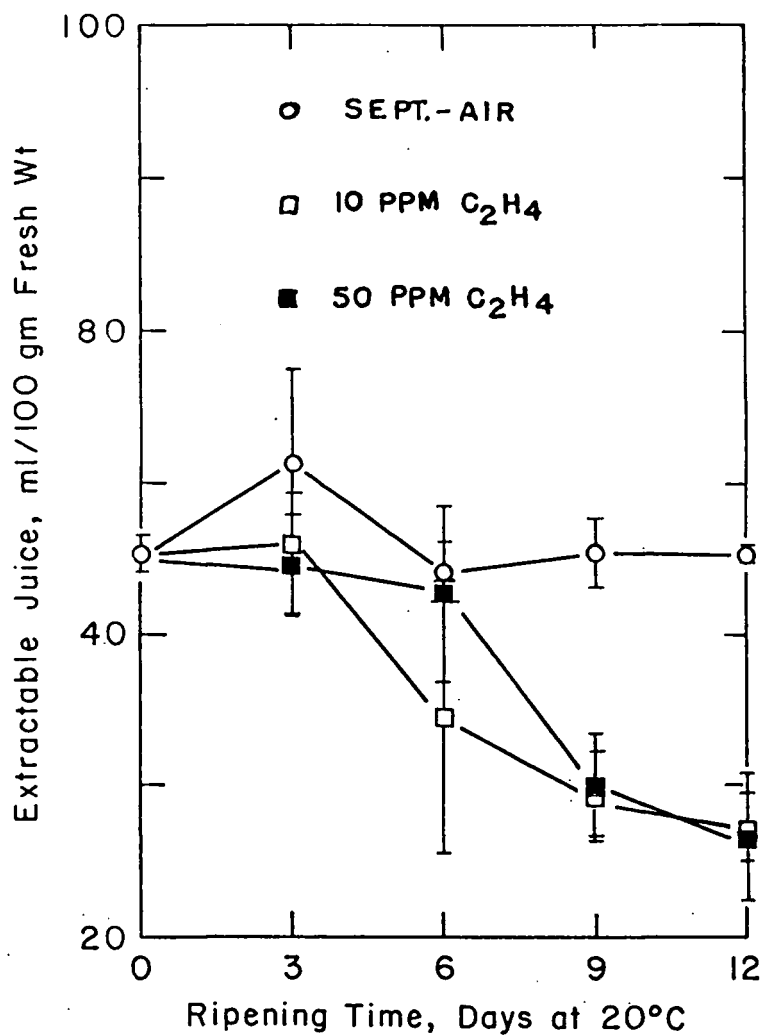


Fig. IV.3. Extractable juice from 'd'Anjou' pear flesh during ripening in air, 10 or 50 ppm ethylene.



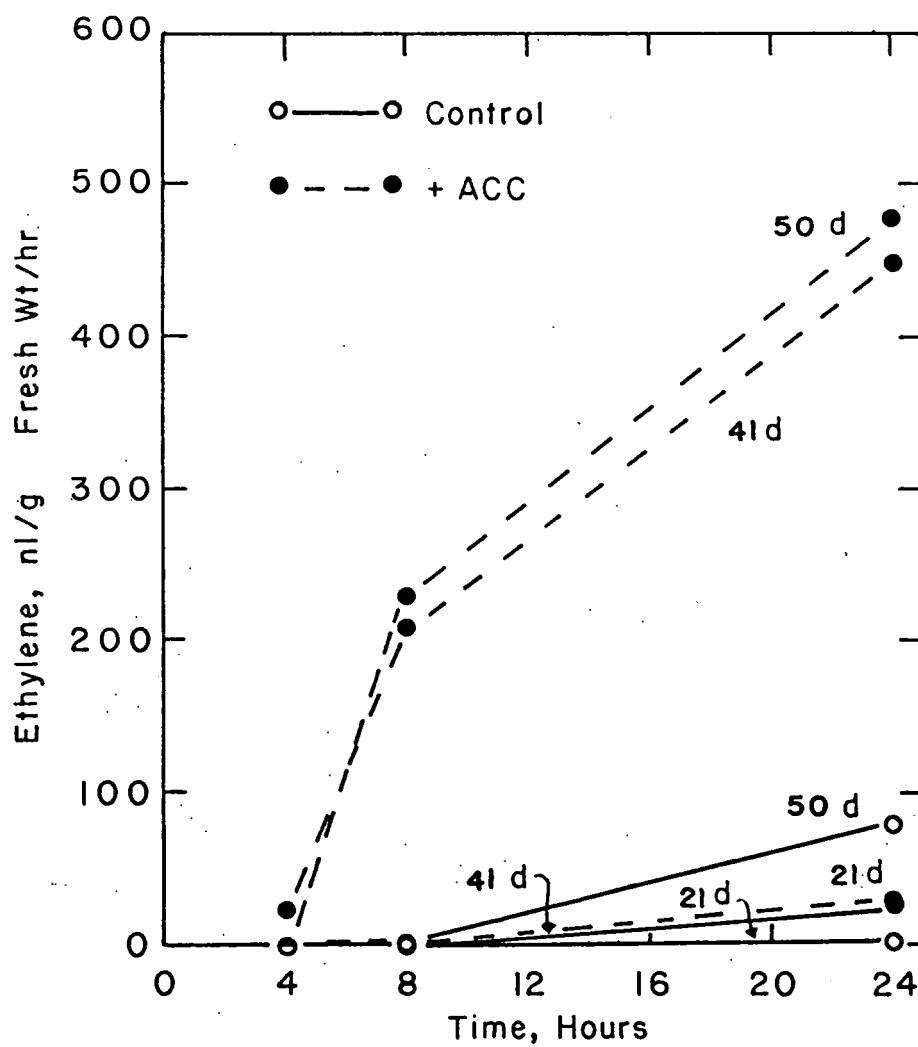


Fig. IV.4. Ethylene production at 20°C by 'd'Anjou' pear discs in the presence or absence of ACC after storage of fruit for 21, 41, or 50 days at -1.1°C.

Table IV.1. Taste panel evaluations of pears capable (November) and incapable (September) of ripening and ethylene treated pears. 1 = sour, 5 = sweet: 1 = dry, 5 = juicy: 1 = coarse texture, 5 = buttery texture: 1 = unripe, 5 = very ripe: 1 = overall very bad, 5 = overall very good.

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QUALITY PARAMETERS					
Treatment	sour- sweet	dry- juicy	coarse- buttery	ripe- ness	over- all
<hr/>					
Unripe- (Sept.)	2.17 ab	1.67 a	1.67 a	1.58 a	1.75 a
Air- (Sept.)	2.00 a	1.33 a	1.75 a	1.58 a	1.75 a
10 ppm C <sub>2</sub> H <sub>4</sub> (Sept.)	3.58 d	4.25 c	3.83 b	3.67 b	3.79 c
50 ppm C <sub>2</sub> H <sub>4</sub> (Sept.)	3.42 d	3.25 b	3.25 b	3.25 b	2.96 b
Air- (Nov.)	2.75 bc	2.96 b	3.21 b	3.04 b	3.08 b
10ppm C <sub>2</sub> H <sub>4</sub> (Nov.)	3.17 cd	4.21 c	3.46 b	3.58 b	3.75 c

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Mean separation in columns by Duncan's multiple range, 5% level.

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## CHAPTER V.

Title: Characteristics and control of ethylene production in pears in air and modified atmosphere storage.

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Additional index words. Pyrus communis, low oxygen storage, 1-aminocyclopropane-1-carboxylic acid, aminoethoxyvinylglycine, maturity, ripening, postharvest physiology.

Abstract. Storage life of 'd'Anjou' pear fruit (Pyrus communis L.) is extended when fruit is held in 1% or 3% O<sub>2</sub> at -1° C, however the mechanism by which low oxygen levels extends fruit life is unclear. 'd'Anjou' pear flesh discs show no ethylene production over 70 h when treated with 0.05 mM cycloheximide. If given 0.5 mM 1-aminocyclopropane-1-carboxylic acid (ACC) in the presence of cycloheximide, discs were able to produce twice the amount of ethylene as a control without exogenous ACC. However, discs with added ACC could produce 4 times the amount of ethylene as discs with exogenous ACC and cycloheximide. Cytoplasmic protein synthesis is apparently required for the synthesis of ACC and the conversion of ACC to ethylene if prolonged or high ethylene production is to occur. Chloramphenicol had no effect on ethylene production. Pears stored for 125 days in 3% O<sub>2</sub> or 153 days in 1% O<sub>2</sub> storage had internal ethylene levels equal to those of air

stored fruit. Endogenous ACC levels were 20 times higher in fruit stored in 1% or 3% O<sub>2</sub> when compared to air stored fruit. When 0.05 mM cycloheximide and 0.5 mM ACC were exogenously supplied to discs cut from pears stored in air or 1% O<sub>2</sub>, the air stored fruit was able to produce 400 times the amount of ethylene as 1% O<sub>2</sub> fruit. This appeared to be due to an effect of oxygen on some other physiological process, rather than a direct limitation of the conversion of ACC to ethylene. Aminoethoxyvinylglycine (AVG) applied as an orchard spray caused a 3 fold reduction in ACC in 'Bosc' pears, but had no significant effect on 'd'Anjou' pears after approximately 200 days air storage at -1°.

## INTRODUCTION

Control and development of the ethylene system in climacteric fruits is of economic interest as the amount of ethylene production has a direct bearing on the length of storage. This is particularly important for pears. Protein synthesis has been shown to be a requirement for ripening in pears and cycloheximide (a cytoplasmic protein synthesis inhibitor), can inhibit ethylene production and ripening (9).

Concentrations of 1-aminocyclopropane-1-carboxylic acid (ACC) have been shown to correlate with peak ethylene production in the climacteric fruits, avocado, banana, and tomato with high ACC levels preceding high ethylene levels (10). The conversion of S-adenosyl methionine to ACC by ACC synthase is generally considered the rate limiting step in ethylene biosynthesis in plants (19). AVG inhibits at the ACC synthase step (3,20). Many plant tissues, producing no ethylene, nevertheless have the ability to convert ACC to ethylene (6). Wound ethylene increased the activity of the system converting ACC to ethylene in cantaloupes (11).

Ethylene production requires oxygen which may be limiting in the conversion of ACC to ethylene (2,19). Reduced oxygen storage prolongs the storage life of fruit. Fruit in reduced oxygen storage produced threshold limits of ethylene (8,13), and once removed from storage ripens more quickly than air stored fruit (7).

The first objective was to determine if the protein synthesis required for ripening and ethylene production was for the synthesis of ACC, the system converting ACC to ethylene, or both. The second objective was to examine ACC levels and ethylene production in 1% and 3% O<sub>2</sub> stored fruit in an effort to understand the mechanism of modified atmosphere storage on ethylene production, and the ethylene pathway. We also measured ACC levels in AVG treated fruit to determine to what extent ACC levels would be affected after long term cold storage.



## MATERIALS AND METHODS

Pears were harvested from the Mid-Columbia Experiment Station, Hood River, Oregon, at commercial maturity of 66.7 N (6.8 kg) firmness and stored at  $-1.1^{\circ}$  C. 'Bosc' and 'd'Anjou' pears in the AVG experiment were harvested in Medford, Oregon. Regular controlled atmosphere conditions (3%  $O_2$ ) were at  $-1.1^{\circ}$  and 0.8-1%  $CO_2$  and 2.5-3.5%  $O_2$  in a commercial storage unit. Three weeks at  $-1.1^{\circ}$  elapsed between harvest and establishment of the 3%  $O_2$  conditions, and fruit was removed after 125 days storage. 1%  $O_2$  conditions were at  $-1.1^{\circ}$  and 0.005-0.095%  $CO_2$  and 0.85-0.96%  $O_2$  in a commercial storage unit. Two weeks at  $-1.1^{\circ}$  elapsed between harvest and establishment of low oxygen conditions. Fruit was removed from 1%  $O_2$  conditions after 153 days.

Discs of pear flesh were cut from 'd'Anjou' pears with a No. 6 cork borer (I.D. 1.1 cm) and trimmed to 5 mm thickness. Fruit had been held from 35 to 45 days at  $-1.1^{\circ}$  in the initial experiments or under the specified reduced oxygen conditions for the later experiments. There were 6 discs per 25 ml sealed Erlenmeyer flask, cut from a minimum of 3 pears. There were 5 replications per treatment. Flasks were sealed for the specified length of time at  $20^{\circ}$  and were aired between sampling times. Each flask contained 2 ml of solution that was vacuum infiltrated into the discs. All compounds were in 0.35 M mannitol. Cycloheximide concentration was 0.05 mM which is sufficiently high to block protein synthesis but does not kill the tissue (9).

Chloramphenicol was 5 mM, and, unless otherwise specified, ACC concentration was 0.5 mM. Control treatments had 2 ml of 0.35 M mannitol vacuum-infiltrated.

Ethylene was measured by gas chromatography on an alumina column. ACC was extracted in 9% perchloric acid then assayed by a modification of the method of Lizada and Yang (14) with 4 replications per treatment. For ACC determination 100 g F.W. of pear flesh was ground in a Wareing blender with 100 ml 9% perchloric acid. The extract was vacuum filtered and brought to 250 ml with distilled water. The equivalent of 10 g F.W. was passed through a Dowex 50W-X4 cation exchange column. ACC was eluted from the column with 5 ml of 2 N  $\text{NH}_4\text{OH}$ . The  $\text{NH}_4\text{OH}$  was removed under vacuum. To each 14 ml vial containing the sample, 800  $\mu\text{l}$  distilled water, and 100  $\mu\text{l}$  of 100  $\mu\text{M}$  mercuric chloride were added. The vials were capped with a rubber serum cap and 100  $\mu\text{l}$  5%  $\text{NaOCl}$ :saturated  $\text{NaOH}$  (2:1) was added. The vials were shaken intermittently for 2.5 min. A 1 ml gas sample was withdrawn from the vial and the amount of ACC which had been converted to ethylene was determined by gas chromatography.

AVG (400 ppm) was applied as a preharvest spray. 'Bosc' pears were sprayed 9 days earlier for the September 3 harvest and 18 days earlier for the September 12 harvest. 'd'Anjou' pears were sprayed 1 day before commercial harvest in September.

## RESULTS AND DISCUSSION

Ethylene production in the presence of cycloheximide was inhibited over 70 h, while chloramphenicol did not significantly affect the tissue ethylene when compared to control discs (Fig. V.1). Cycloheximide inhibits cytoplasmic protein synthesis while chloramphenicol inhibits mitochondrial protein synthesis. Cycloheximide has been shown to inhibit the synthesis of enzymes needed for ripening in 'Bartlett' pears, and was found to be less effective as 'Bartlett' fruit approached the climacteric (9). Protein synthesis is apparently necessary for even minimal ethylene production in the absence of exogenous ACC. If given cycloheximide and ACC, discs can produce ethylene significantly higher than the control for a limited length of time. The decline in conversion of added ACC to ethylene after 40 h in the presence of cycloheximide implies turnover of the enzyme(s) converting ACC to ethylene. ACC may be partially limiting, as evidenced by the stimulation of ethylene by added ACC (Fig. V.2). There is most likely some ability to convert ACC to ethylene normally present in the tissue at this stage since cytoplasmic protein synthesis would be blocked and any new synthesis prevented. Several other plant tissues have been shown to possess the ability to convert exogenous ACC to ethylene even though they naturally produce no ethylene (6).

Immediate infiltration of cycloheximide resulted in no ethylene production (Fig. V.3). Infiltration after 30 or 60 min.

resulted in only minimal ethylene production. Infiltration after 2 h allowed some ethylene production to take place. The control produced much higher amounts of ethylene than the cycloheximide treatments indicating that constant protein synthesis is necessary for high and prolonged ethylene synthesis. It's interesting to note that there was still increasing ethylene production up to 20 h despite only 2 hours of protein synthesis allowed before the infiltration of the cycloheximide. It implies that turnover of the ACC to ethylene enzymes(s) is not extremely fast.

In the presence of ACC, time lapses of 10, 30 or 60 min. before cycloheximide infiltration did not allow the discs ample time for protein synthesis such that ethylene production was stimulated (Fig. V.4). The control initially didn't produce ethylene, presumably due to lack of ACC. If given ACC, even in the presence of cycloheximide, ethylene production began immediately. The levels of endogenous ACC and ACC synthase have been suggested as the rate limiting step in plant tissue (1). Wound ethylene has been shown to be regulated at ACC synthase (4) and wound ethylene will apparently increase the activity of the ACC to ethylene system (11). Two hours of incubation before infiltration of cycloheximide was needed in order to utilize the added ACC. De novo synthesis of the enzyme to convert ACC to ethylene or some other factor needed for this step was probably taking place. It is currently thought that the conversion of ACC to ethylene may involve a free radical reaction or indirectly a peroxidase reaction (17).

One of two situations seems to exist. First, if ACC is limiting in the control discs, the tissue should be able to convert added ACC. In fact it can do so without any problem (Fig. V.2) and this seems to imply that the enzyme that converts ACC to ethylene is plentiful. Under this situation it would seem logical that ACC synthase was the limiting step has been shown in other plant tissues (1,6). The second possibility is that ACC synthesis is not the limiting step. Cycloheximide treated discs with ample exogenous ACC can't make any new ACC to ethylene enzyme(s) or any cofactors by cytoplasmic protein synthesis. The imposed limiting factor on these discs would be the amount of the enzyme(s) converting ACC to ethylene or some other needed cofactor. However, the cycloheximide and ACC discs cannot produce any more ethylene than the control discs without the exogenous ACC that was assumed to be limited by the amount of ACC present. This implies that the amount of ACC was not the limiting factor in the control discs after all, but the limit was perhaps a third factor which high levels of ACC might stimulate if protein synthesis is allowed to continue normally.

Internal ethylene levels in air stored fruit held 125 days or 153 days, 3% O<sub>2</sub> stored fruit after 125 days, or 1% O<sub>2</sub> stored fruit held 153 days had equivalent internal ethylene production on removal from storage (Fig. V.5). 1% O<sub>2</sub> stored fruit had

significantly higher ethylene production during ripening. In apples (13) and 'd'Anjou' pears (8), low oxygen concentrations also failed to prevent threshold ethylene levels.

ACC levels were 20 times as higher in 1%  $O_2$  or 3%  $O_2$  stored pears when compared to air stored pears when fruit was first removed from cold storage (Fig. V.6). It took 6 days at 20°C to equalize ACC levels. The higher amounts of ACC in 3%  $O_2$  fruit were apparently converted slowly to ethylene, or changed in some other way as no apparent difference in ethylene production existed (Fig. V.5). Fruit from 1%  $O_2$  storage also had higher levels of ACC than air stored fruit. Pears held in reduced oxygen storage had accumulated large pools of ACC, which was slowly converted to ethylene as opposed to a rapid conversion to ethylene upon removal from low oxygen conditions to air at 20°. The large pool of ACC present in low oxygen stored fruit remained in the fruit for several weeks at -1.1° after removal from low oxygen conditions (data not shown). The observation that the ethylene pathway in apple can develop in low oxygen but is more active under high oxygen (13) is consistent with the present result of accumulation of ACC in pears in 1%  $O_2$  storage. The conversion of ACC to ethylene requires oxygen (2,19). However, low oxygen storage does not prevent accumulation of threshold concentrations of ethylene (8,13) and the beneficial results of reduced oxygen cannot be solely ascribed to limiting the step of ACC to ethylene despite

the accumulation of ACC (12,13). Upon subjecting apples to a change in atmosphere, internal atmospheres equilibrate with respect to  $O_2$  of the external atmosphere in 6-16 hours (13).

Differences in fruit firmness were minimal between 3%  $O_2$  and air stored fruit. However, 1%  $O_2$  stored fruit softened to 18 N compared to air stored fruit at 31 N after 6 days ripening at 20°C (data not shown). Upon ripening 1%  $O_2$  stored pears have been observed to soften and achieve dessert quality faster than air stored pears (7). The higher levels of internal ethylene and larger pools of ACC in 1%  $O_2$  stored fruit could account for the faster ripening observed in this fruit.

When pear flesh discs from 1%  $O_2$  stored pears were given cycloheximide and exogenous ACC they could not produce as much ethylene as discs from air stored pears (Fig. V.7). In the absence of cycloheximide, but with added ACC, discs from low oxygen pears did not produce as much ethylene as discs from air stored pears. The capacity of low oxygen fruit to make the necessary enzymes or cofactors appears to be less than in air stored fruit. Low oxygen conditions could lower protein synthesis, metabolic rates or energy charge (13).

AVG has been shown to prevent ethylene production in pears (15,16,18) and apples (5). AVG inhibits ACC synthase in isolated enzyme systems (3,20). When ACC levels in fruit treated with AVG

were measured late in the storage season of 'd'Anjou' and 'Bosc' pears, there is an accumulation of ACC in 'Bosc' and to a lesser extent in 'd'Anjou' (Fig. V.8). AVG apparently has the same effect in intact fruit as in isolated systems.



## CONCLUSIONS

Cytoplasmic protein synthesis is required for high or prolonged ethylene synthesis, both for the synthesis of ACC and the system converting ACC to ethylene. This indicates that either step could potentially control ethylene production. Fruit stored in reduced oxygen atmospheres has substantially more ACC than air stored fruit. Under reduced oxygen conditions the conversion of ACC to ethylene becomes the limiting step in ethylene production rather than ACC synthesis. This limitation is not solely due to the direct effect of oxygen on the conversion of ACC to ethylene. AVG reduced the amount of ACC in stored 'Bosc' pears and the effect was evident for the duration of the useful life of the pear.

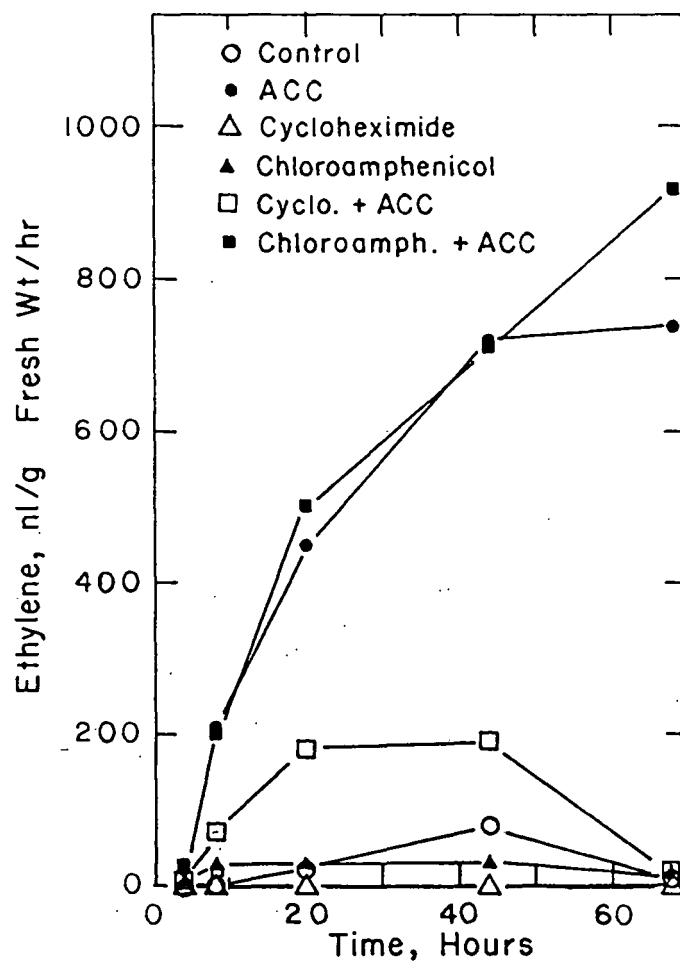


Fig. V.1. Effect of 0.05 mM cycloheximide and 5 mM chloramphenicol with or without 0.5 mM ACC on ethylene production in tissue discs from 'd'Anjou' pears stored 35 days at  $-1.1^{\circ}\text{C}$ .

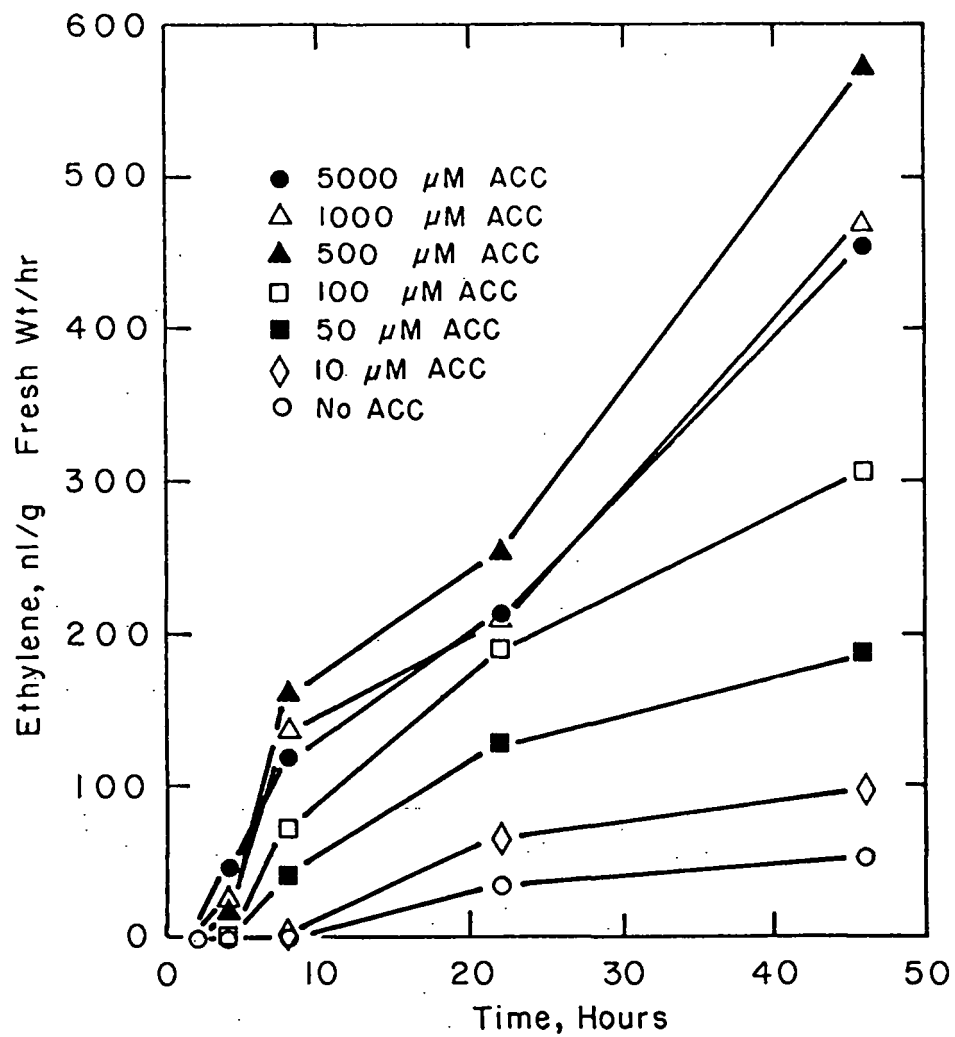


Fig. V.2. Effect of ACC concentration on ethylene production in tissue discs from 'd'Anjou' pears stored 45 days at  $-1.1^{\circ}\text{C}$ .

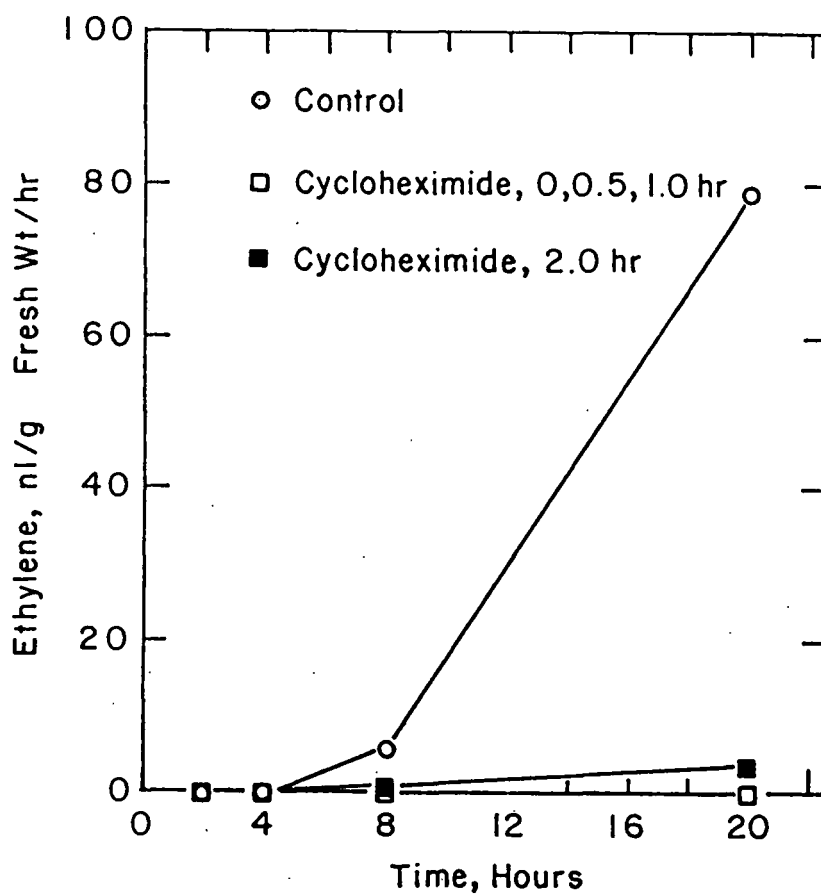


Fig. V.3. Effect of 0.05 mM cycloheximide infiltrated after 0, 10, 30, 60 or 120 minutes on 'd'Anjou' pear tissue disc ethylene production after 42 days cold storage at  $-1.1^{\circ}\text{C}$ .

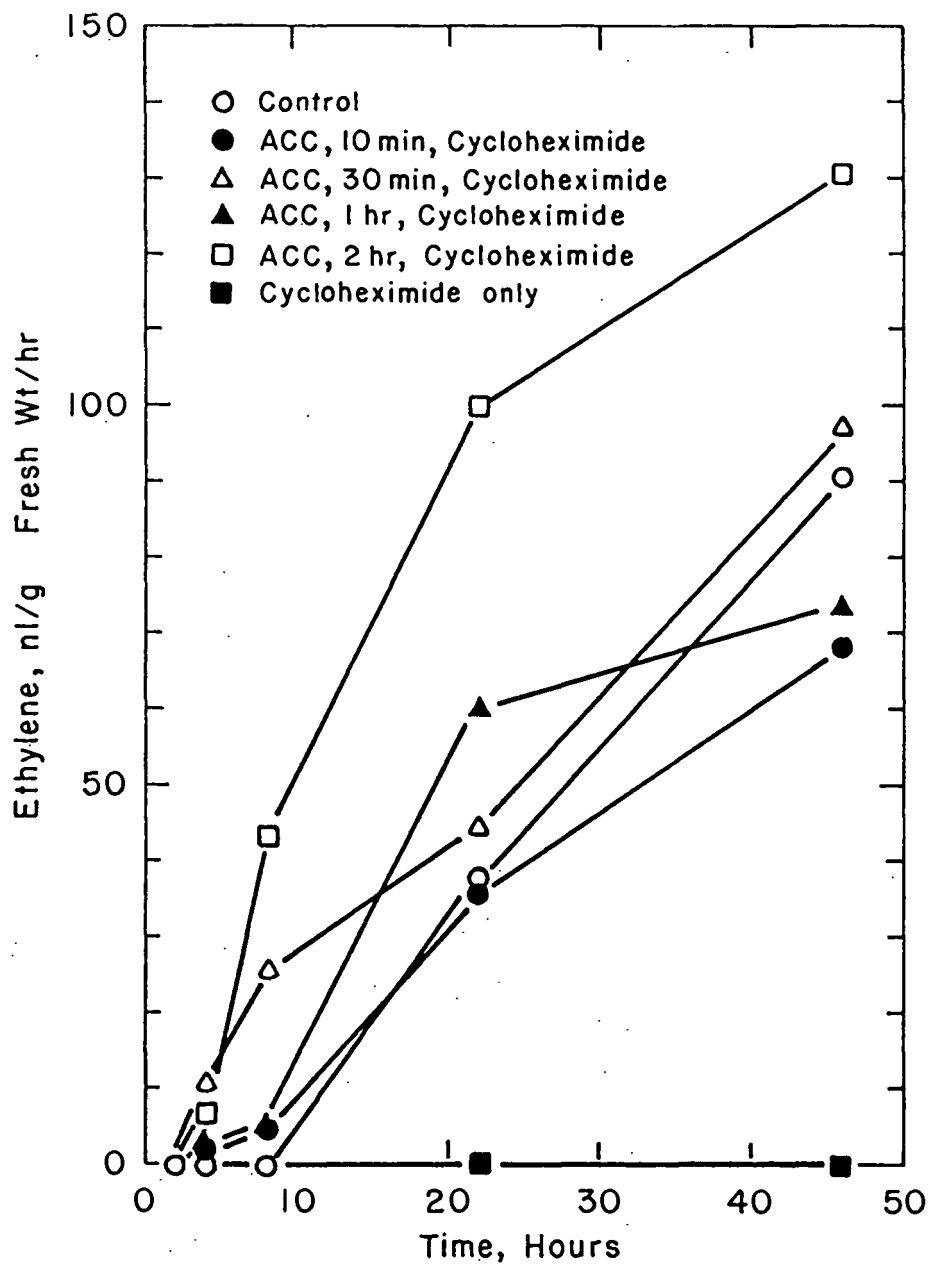


Fig. V.4. Effect of 0.05 mM cycloheximide infiltration after 0, 10, 30, 60 or 120 minutes on 'd'Anjou' pear disc ethylene production in the presence of 0.5 mM ACC after 42 days cold storage at  $-1.1^{\circ}\text{C}$ .

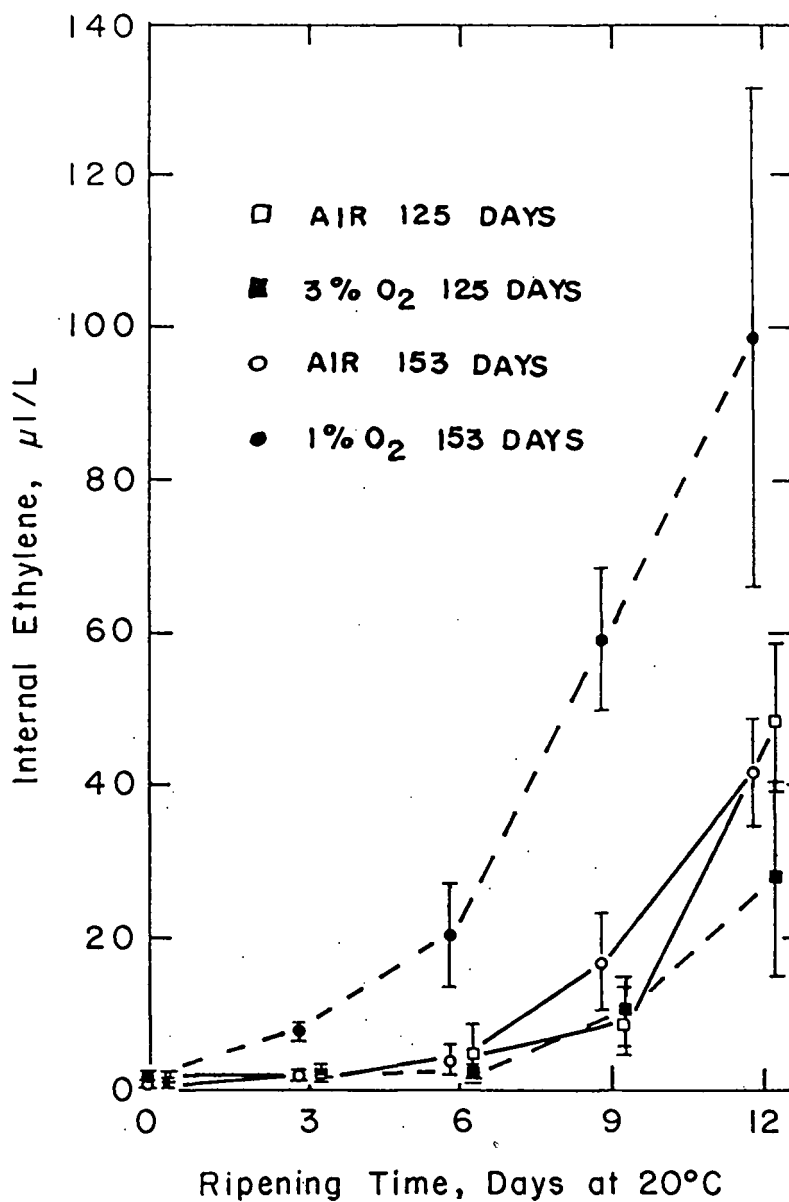


Fig. V.5. Internal ethylene during ripening of 'd'Anjou' pears that had been held 125 days in air or 3%  $\text{O}_2$ , or 153 days in air or 1%  $\text{O}_2$ .

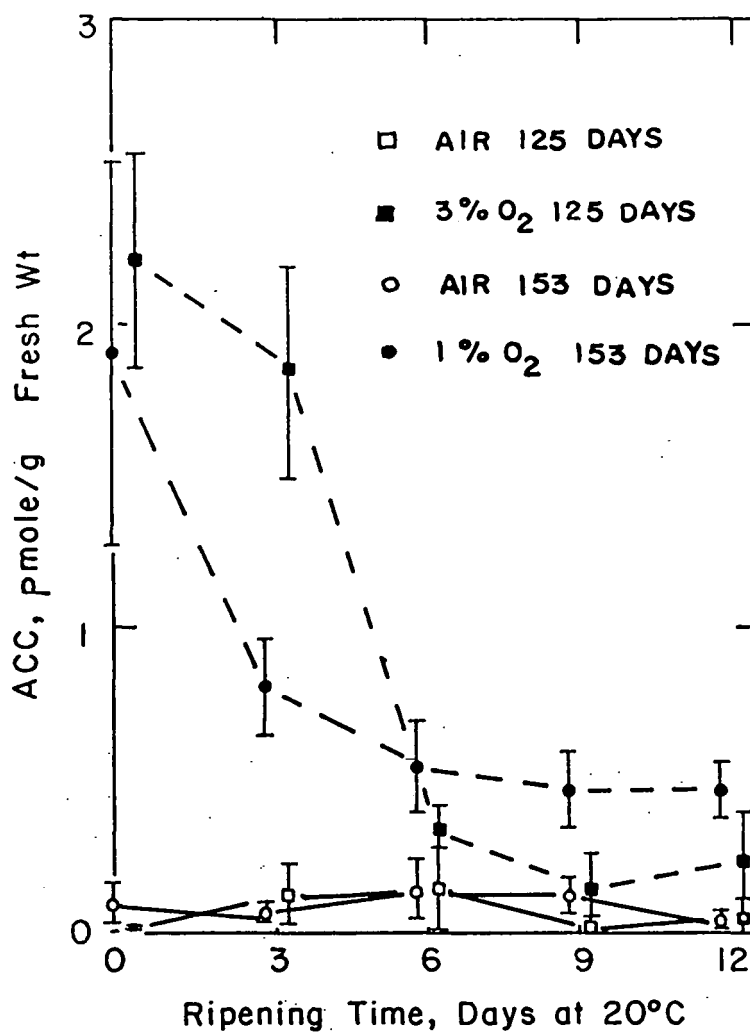


Fig. V.6. ACC during ripening of 'd'Anjou' pears that had been held 125 days in air or 3% O<sub>2</sub>, or 153 days in air or 1% O<sub>2</sub>.

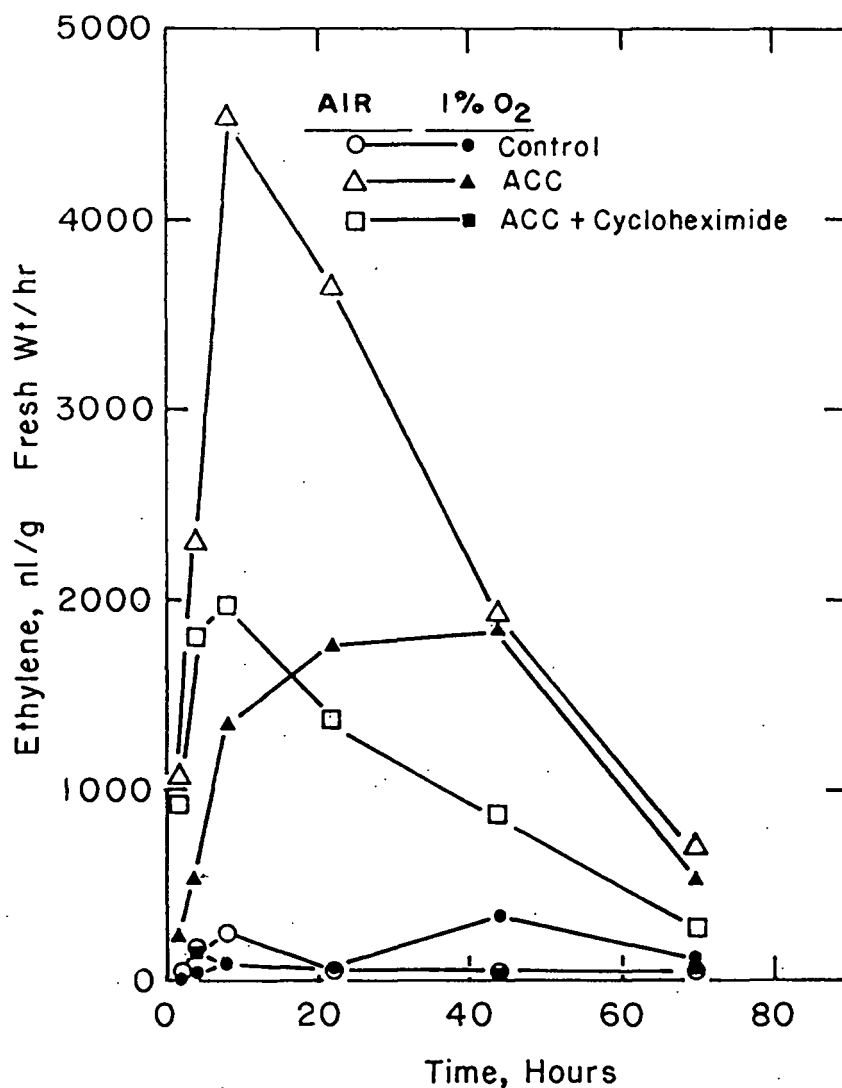


Fig. V.7. Effect of 0.05 mM cycloheximide in the presence or absence of 0.5 mM ACC on ethylene production in 'd'Anjou' pear discs cut from fruit stored 153 days in air or 1% O<sub>2</sub> at -1.1°C. Control discs were in 0.35 M mannitol.



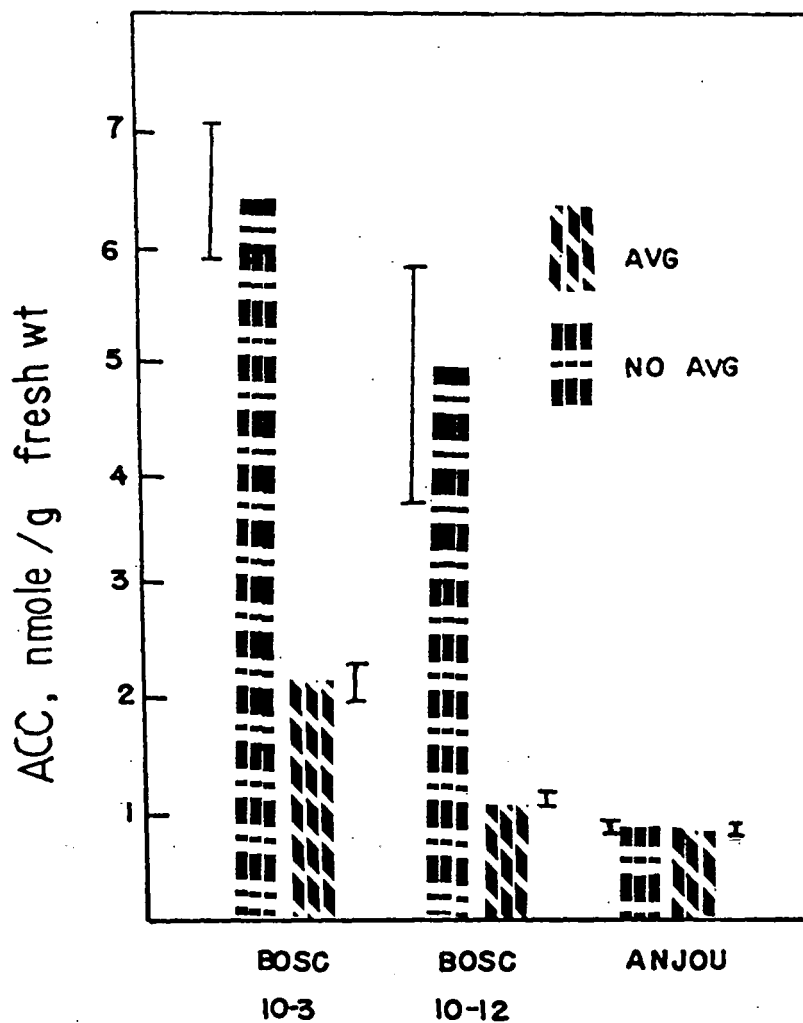


Fig. V.8. Effect of 400 ppm preharvest spray of AVG on ACC levels in 'Bosc' pears harvested October 3 or 12, and 'd'Anjou' pears harvested September 16, and stored for 200, 209 or 218 days, respectively, at  $-1.1^{\circ}\text{C}$ .

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## CHAPTER VI.

## CONCLUSIONS

During cold storage and subsequent ripening of pears major biochemical changes take place which ultimately influence the quality and storage life of pears. Long-term cold storage increases chlorogenic acid, catechin, epicatechin and p-coumaroyl quinate. Bruising increases chlorogenic acid and catechin. Epicatechin, p-coumaroyl quinate, and a p-coumaric acid derivative appear with the initiation of ethylene production, however no definite link was established between ethylene synthesis and phenolic acids.

During the cold storage period, which is necessary to stimulate sufficient ethylene to cause ripening of pears, exogenous ethylene will cause a slight respiratory rise, a decline in fruit firmness, and the pears are perceived to be juicier and sweeter by a taste panel. The cold period necessary for ripening initially allows the ability to convert ACC to ethylene to develop, and later the ability to synthesize ACC. While it has been generally believed that the synthesis of ACC is the limiting step in ethylene biosynthesis, it appears that there may also be earlier regulation at the conversion of ACC to ethylene. This offers another possible step to control ethylene production in pears and the ensuing deterioration.

The mechanism of how controlled atmosphere storage retards fruit senescence seems to be at a basic level. ACC levels increase in low oxygen storage, but ethylene production is sufficiently high to cause ripening. There appears to be less of the system that converts ACC to ethylene in low oxygen stored pears. A preharvest spray of AVG will cause a decrease in ACC levels in stored pears.

It is hoped that as biochemical and physiological changes in harvested pears are defined in various situations, that mechanisms to control the aging process in pears can be developed, resulting in a higher quality product.

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## APPENDICES

The phenolic acid analysis methods used to derive the information in Fig. A.1 and A.2 are described in Chapter III. 'd'Anjou' pears were ripened for 12 days at 20°C. The modified atmosphere storage conditions are described in Chapter V.

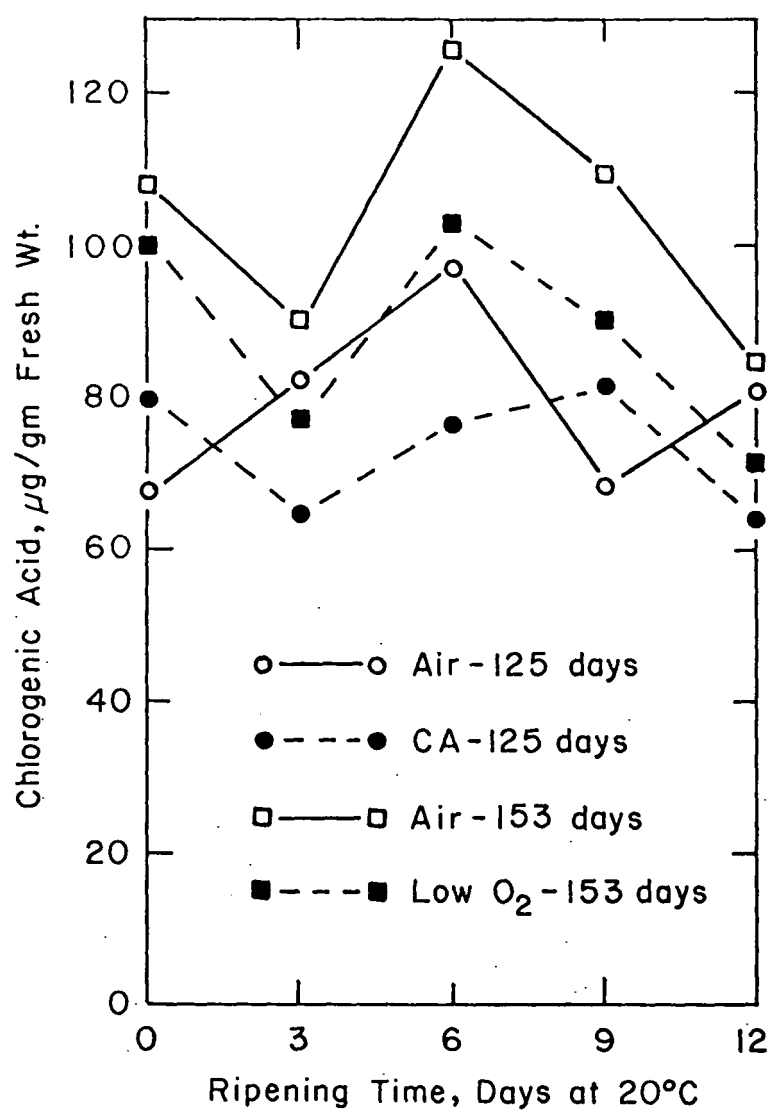


Fig. A.1. Chlorogenic acid levels during ripening of 'd'Anjou' pear flesh after storage at  $-1.1^{\circ}\text{C}$  for 125 days in air or 3%  $\text{O}_2$  or 153 days in air or 1%  $\text{O}_2$ .

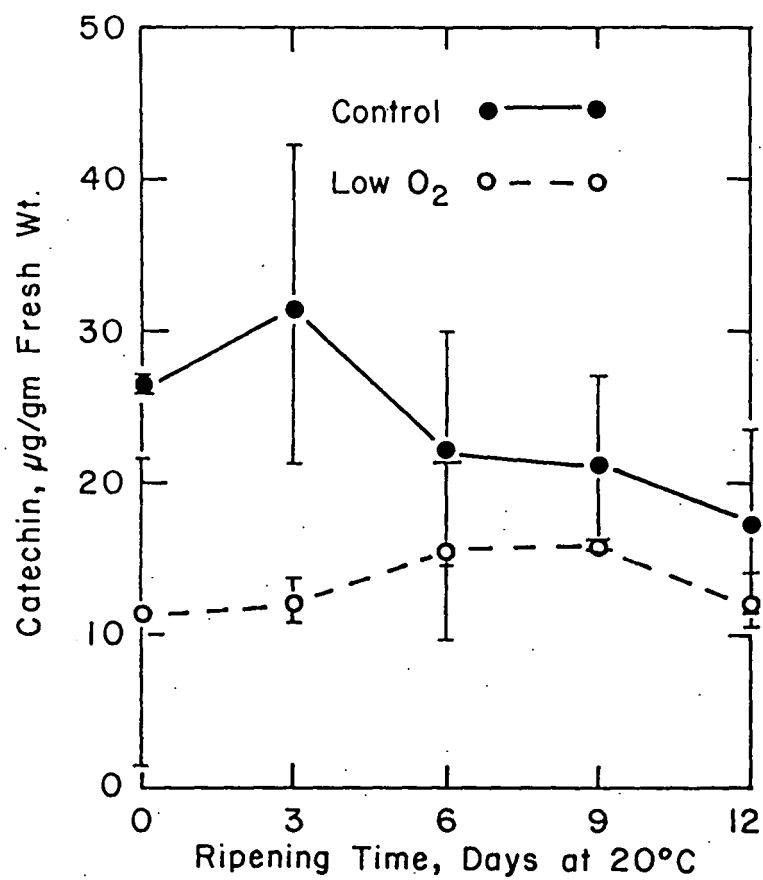


Fig. A.2. Catechin levels during ripening of 'd'Anjou' pears after storage at  $-1.1^\circ\text{C}$  for 153 days in air or 1%  $\text{O}_2$ .



The following procedures were used to arrive at the information in Fig. B.1. Extraction and assay procedures for pear inhibitors to fungal polygalacturonase have been described (7,8). 100 g pear flesh (33 g from each of 3 pears) was ground in 100 ml 0.1 M sodium acetate buffer, pH 6.0, containing 0.2% (w/v) sodium dithionite and 1% (w/v) PVP (M.W. 40,000). The homogenate was degassed and centrifuged at 10,000 g for 30 min at 4°C. The supernatant was frozen and is referred to as Fraction I. The residue was suspended in 75 ml 1 M sodium acetate containing 6% (w/v) sodium chloride, which was adjusted to pH 8.2. The mixture was allowed to sit at 4.4°C for 4 h and was shaken occasionally. It was then centrifuged at 10,000 g for 15 min at 4°C. The supernatant was removed and saved and the residue was washed with 25 ml 1 M sodium acetate containing 6% sodium chloride. After centrifuging a second time, the wash and first salt extract were combined and frozen. This fraction is referred to as Fraction II. The fractions were dialyzed (M.W. cutoff 6,000-8,000) at 4.4°C for 24 h. Fractions were assayed by the cup-plate method on a plate with a surface of 23 cm X 23 cm, a gel thickness of 4 mm, with 8 mm diameter holes cut with a No. 3 cork borer. The gel was composed of 250 ml 0.1 M. sodium acetate buffer, pH 5.0, 2.5 g sodium polypectate, 1.25 g ammonium oxalate, and 3.75 g Bacto-agar, which was blended then autoclaved. The dialyzed fractions were pipetted into the holes cut in the gel plate in the presence or absence of Aspergillus niger polygalacturonase (Sigma Chemical Co., St. Louis, Missouri). The plates were allowed to develop for

16 h at 40°. A thin layer of 3 N HCl was spread over the surface of the plate to develop the white precipitated rings. The outside diameter of the rings were measured for standard Aspergillus niger polygalacturonase and in the presence of Fraction I or II, and the % inhibition of the fungal polygalacturonase was calculated. Duplicate composite samples of 3 fruits were assayed for each treatment.

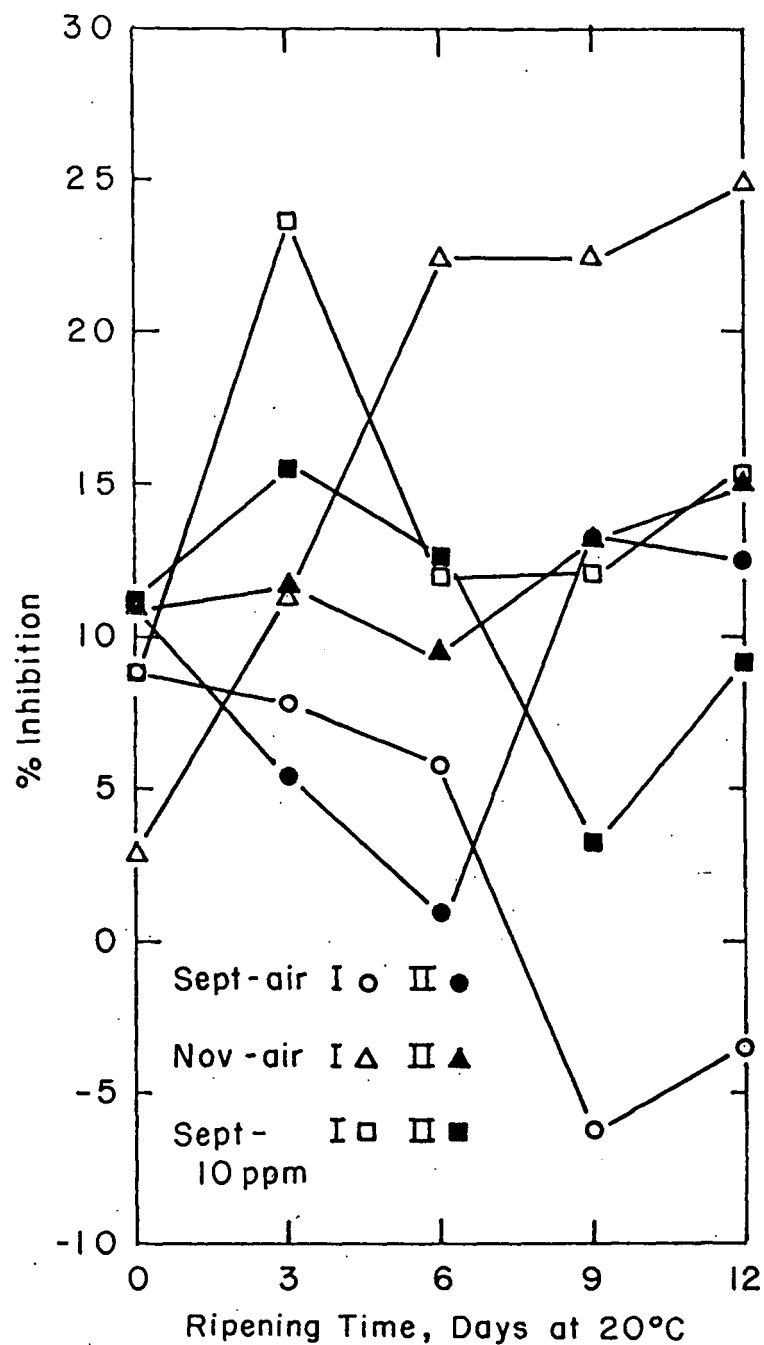


Fig. B.1. Changes in a 'd'Anjou' pear inhibitor to *Aspergillus niger* polygalacturonase during ripening in air in September, air in November, or 10 ppm ethylene in September. I = Fraction I. II = Fraction II.